DRUG TRANSPORT AND DRUG-DRUG INTERACTIONS AT THE BLOOD-BRAIN BARRIER

Thesis submitted in accordance with the requirements of
the University of Liverpool for the degree of
Doctor in Philosophy

by

Steffen Rädisch

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is it currently being presented, either wholly or in part for any other degree or qualification.

Steffen Rädisch

The research presented in this thesis was carried out in the Wolfson Centre for Personalised Medicine, Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool, United Kingdom

and

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany

Contents

Abstract

Membrane transporters are increasingly recognised as being important in determining drug pharmacokinetics at whole body, organ, and cellular levels. At the blood-brain barrier (BBB), membrane transporters determine the passage of drugs into and out of the brain. About 30 % of all patients are classed as non-responders for both epilepsy and schizophrenia. Drug transporters from the adenosine 5'-triphosphate (ATP)-binding cassette (ABC) transporter family or from the solute carrier (SLC) superfamily may contribute to these drug resistant phenotypes but most have received limited attention.

Treatment response to carbamazepine (CBZ) has been associated with genetic polymorphisms in *ABCC2*, particularly -24C>T, c.1249G>A, and c.3972C>T. However, the results have been conflicting and inconclusive amongst the different studies. A functional and clinical analysis was undertaken to investigate the impact of ABCC2 on CBZ treatment response. *In vitro*, no ABCC2-mediated CBZ transport could be observed in efflux assays with an *ABCC2*-transfected human fibrosarcoma cell line (Rht14-10) and a dog kidney cell line (MDCKII). In addition, uptake into inside-out vesicles derived from the Rht14-10 cell line was negative. Clinical analysis of patients from the SANAD (Standard and New Antiepileptic Drugs) trial (assessing the clinical end-points time to first seizure (n = 229) and time to 12-month remission (n = 134)) did not show any significant associations between the three *ABCC2* gene polymorphisms, -24C>T, c.1249G>A, c.3972C>T, and clinical outcomes.

In an attempt to identify currently unrecognised human drug transporters with potential relevance to epilepsy and schizophrenia, screening of transport of CBZ, lamotrigine (LTG), topiramate (TPM), levetiracetam, valproate, phenytoin, and clozapine (CLP) was undertaken using an immortalised human brain endothelial cell line (hCMEC/D3) as an *in vitro* model of the BBB. Accumulation of TPM was significantly enhanced by 44-53 % in the presence of the typical ABCC efflux transporter inhibitors MK571 and montelukast. Furthermore, CLP uptake was significantly reduced by 94 % and 83 % in the presence of the typical organic cation transporter inhibitors prazosin and verapamil, respectively. CLP uptake into the hCMEC/D3 cell line followed classical Michaelis-Menten kinetics with V_{max} of 3288 (pmol/million cells)/min and K_m of 35.93 µM. To identify the exact underlying transporters involved in TPM efflux and CLP uptake, both functional siRNA screening was undertaken and transport was investigated in transfected cell lines. None of the known functional ABCC transporters were shown to transport TPM. In addition, none of the expressed and functionally characterised organic cation transporters from the SLC22A family, as well as transporters from the SLC6A, SLC28A, and SLC29A families, had an effect on CLP accumulation.

LTG has recently been identified as a substrate for SLC22A1 (OCT1). Interaction with the human immunodeficiency virus protease inhibitors lopinavir/ritonavir and the antipsychotic CLP was therefore investigated. At clinically relevant concentrations, lopinavir was found to significantly reduce SLC22A1-mediated uptake of LTG by 39 %. In addition, CLP was a potent inhibitor of SLC22A1-mediated LTG uptake yielding an IC_{50} of 1.8 µM. Similarly low IC_{50} values were obtained with primary human hepatocytes from two patients (IC_{50} = 7.9 µM and IC_{50} = 3.9 µM, respectively) and the hCMEC/D3 cell line (IC_{50} = 2.0 µM). The

clinical consequences of these observations will require further *in vivo* pharmacokinetic and epidemiological research.

In conclusion, the results presented in this thesis demonstrate that membrane transporters can be involved in the passage of AEDs and antipsychotics across the BBB and other membrane barriers. However, currently available *in vitro* methods proved to be insufficient to identify and characterise the underlying transporters involved and to further evaluate the impact on treatment efficacy. The development of large-scale functional screening methodologies will be crucial for a more systematic and comprehensive understanding of drug transport processes involved in determining access of drugs to the central nervous system. This will help in improving drug efficacy and drug safety, allow prediction of drug-drug interactions, and eventually allowed a more personalised approach to prescribing in diseases such as epilepsy and schizophrenia.

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Publications

Rädisch S, Dickens D, Lang T, Bonnett L, Arlanov R, Johnson MR, Schwab M, Marson AG, Pirmohamed M (2014). A comprehensive functional and clinical analysis of ABCC2 and its impact on treatment response to carbamazepine. *The Pharmacogenomics Journal*. Advance online publication 25 February 2014

Dickens D, Webb SD, Antonyuk S, Giannoudis A, Owen A, **Rädisch S**, Hasnain SS, Pirmohamed M (2013). Transport of gabapentin by LAT1 (SLC7A5). *Biochemical Pharmacology* **85**. 1672-1683

Manuscripts in preparation

Alfirevic A, Durocher J, Elati A, Leon W, Dickens D, **Rädisch S**, Box H, Siccardi M, Curley P, Xinarianos G, Ardeshana A, Owen A, Zhang JE, Pirmohamed M, Alfirevic Z, Weeks A, Winikoff B. Misoprostol-induced fever and genetic polymorphisms in drug transporters *SLCO1B1* and *ABCC4* in women of Latin American and European ancestry

Oral presentations

Rädisch S, Dickens D, Pirmohamed M. Antiepileptic drug transport at the blood-brain barrier. *Fighting Drug Failure Network Meeting.*
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23 November 2012, Liverpool, UK

Rädisch S, Dickens D, Lang T, Arlanov R, Schwab M, Pirmohamed M. Carbamazepine is not a substrate for ABCC2. *British Pharmacological Society Winter Meeting*.
19 December 2012, London, UK

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Abbreviations

AAE	Avascular arachnoid epithelium
ABC	ATP-binding cassette
ACTB	Beta-actin
ADME	Absorption, distribution, metabolism, and excretion
AED	Antiepileptic drug
AMP	Adenosine 5'-monophosphate
ANOVA	Analysis of variance
APD	Antipsychotic drug
ASP^+	4-(4-(Dimethylamino)styryl)-N-methylpyridinium
ATP	Adenosine 5'-triphosphate
AUC	Area under the curve
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BCSFB	Blood-cerebrospinal fluid barrier
BEC	Brain endothelial cell
bFGF	Fibroblast growth factor-basic (amino acids 1-155) recombinant human protein
BGB	Bisglucuronosyl bilirubin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor
CBZ	Carbamazepine
CDCF	5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate mixed isomers
cDNA	Copy DNA
CEU	Utah residents with northern and western European ancestry
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
CLP	Clozapine

CMFDA	5-chloromethylfluorescein diacetate
CNS	Central nervous system
CNT	Concentrative nucleoside transporter
cphBEC	Cultured primary human brain endothelial cell
CSF	Cerebrospinal fluid
C_t	Threshold cycle
Cyclo(his-pro)	Histidyl-proline diketopiperazine
CYP	Cytochrome P450
DHEAS	Dehydroepiandrostone sulfate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DPM	Disintegrations per minute
$E_2$17βG	Estradiol-17β-D-glucuronide
E3S	Estrone-3-sulfate
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ENT	Equilibrative nucleoside transporter
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGD	First generation antipsychotic drug
fmBEC	Freshly isolated mouse brain endothelial cell
5-FU	5-Fluorouracil
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBP	Gabapentin
GoI	Gene of interest
GS-MF	Glutathione-methylfluorescein
GSH	Glutathione
GWAS	Genome-wide association study

hBMEC	Human brain microvascular endothelial cell
HBSS	Hanks' balanced salt solution
hCMEC/D3	Human microvascular brain endothelial cell line D3
HEK	Human embryonic kidney cells
HGNC	Human Genome Organisation Gene Nomenclature Committee at the European Bioinformatics Institute
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hPSC	Human pluripotent stem cell
HS	Hippocampal sclerosis
IC_{50}	Half-maximum inhibition
IND	Investigational new drug
ITC	International transporter consortium
ITS	Insulin-transferrin-selenium
JAM	Junctional adhesion molecule
KCL22	Human chronic myeloid leukaemia cell line
LacY	Lactose permease
LAT	L- alpha amino acid transporter
LD	Linkage disequilibrium
LeuT	Leucine transporter
LEV	Levetiracetam
logD	Distribution coefficient
logP	Partitioning coefficient
LTC_4	Leukotriene C_4
LTG	Lamotrigine
MAF	Minor allele frequency
MDCK	Madin Darby canine kidney cell line
MDR	Multidrug resistance protein
MGB	Monoglucuronosyl bilirubin
MPP^+	1-Methyl-4-phenylpyridinium
MRI	Magnetic resonance imaging

mRNA	Messenger RNA
MRP	Multidrug-resistance associated protein
MTLE	Mesial temporal lobe epilepsy
MTX	Methotrexate
NBD	Nucleotide-binding domain
NEAA	Non-essential amino acids
NRTI	Nucleoside reverse transcriptase inhibitor
NS	Non-significant
NTT	Neurotransmitter transporter
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OCTN	Organic zwitterion/cation transporter
PBS	Phosphate buffered saline without calcium/magnesium
PCR	Polymerase chain reaction
PET	Positron emission tomography
PGE_2	Prostaglandin E_2
Pgp	P-glycoprotein
PHT	Phenytoin
PXR	Nuclear receptor pregnane X
Rht14-10	Human fibrosarcoma cell line
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcriptase polymerase chain reaction
SANAD	Standard and new antiepileptic drugs
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SGD	Second-generation antipsychotic drug
siRNA	Small interfering RNA
SJS	Stevens-Johnson syndrome
SLC	Solute carrier

SNP	Single nucleotide polymorphism
SUR	Sulfonylurea receptor
SV	Synaptic vesicle glycoprotein
TEA^+	Tetraethylammonium
TEER	Transepithelial resistance
TEN	Toxic epidermal necrolysis
TMD	Transmembrane domain
TMH	Transmembrane helix
TPM	Topiramate
TR-	Transport deficient
UGT	Uridine 5'-diphospho-glucuronosyltransferase
US	United States
VPA	Valproic acid
WT	Wildtype
ZO	*Zonula occludens*

Chapter 1

General introduction

Contents

1.1. The blood-brain barrier

1.1.1. Structure and function

The central nervous system (CNS) is of fundamental importance for the control and regulation of physiological processes. It is an extremely sensitive microenvironment and thus strict homeostatic regulation is essential in order to maintain neuronal signalling. A key aspect in regulation is the physical separation of the CNS from the rest of the body by means of CNS barriers. CNS barriers restrict the access, but also the exit, of molecules into and from the brain and thereby provide control over concentration and composition of ions, neurotransmitters, macromolecules, neurotoxins, and nutrients (Abbott *et al.* 2010).

In humans, three CNS barriers are known to exist: the blood-cerebrospinal fluid barrier (BCSFB), the avascular arachnoid epithelium (AAE), and the blood-brain barrier (BBB) (Abbott *et al.* 2010). The BCSFB is the outer epithelial cell layer of the choroid plexus, located in the lateral ventricles (third and fourth) of the brain, separating the cerebrospinal fluid (CSF) from blood. The choroid plexus is a branched structure with various villi reaching into the ventricles, composed of connective tissue and fenestrated “leaky” blood capillaries. It is the main source of CSF that is produced by blood “leaking” through the capillaries and then being filtered by the overlying BCSFB (Brown *et al.* 2004). The AAE is an avascular CNS barrier and, as part of the meninges, completely surrounds the brain (Abbott *et al.* 2010). The meninges consist of three layers, the outer one being referred to as *dura mater*, the middle layer as AAE or *arachnoid mater*, and the inner layer as *pia mater*. AAE and *pia mater* are separated by the sub-arachnoid space, a sponge-like structure that is connected to the ventricles and filled with CSF. The AAE itself consists of several epithelial cell layers with the middle layer forming the barrier (Abbott *et al.* 2010). The BBB is the endothelial cell layer forming the walls of all capillaries in the brain and represents the third and largest CNS barrier with regards to surface area. In contrast to the other two barriers, the BBB directly separates the blood from the extracellular fluid of CNS neuronal tissue and hence is in close

proximity to neurons (Abbott *et al.* 2010). These features make the BBB the most important CNS barrier in pharmacology.

1.1.1.1. Tight junctions

The key attribute of all three discussed CNS barriers is the presence of intercellular tight junctions (*zonula occludens*) (Abbott *et al.* 2010). Tight junctions form an intercellular "seal" and highly restrict any paracellular diffusion of molecules through a cell monolayer. They are part of the junctional complex generally involved in cell adhesion (Farquhar *et al.* 1963). The junctional complex also includes adherence junctions, important structural proteins such as the cadherin-catenin complex, that attach endothelial or epithelial cells and are essential to build-up tight junctions (Abbott *et al.* 2010, Kurita *et al.* 2013). Adherens junctions are located underneath tight junctions which are found at the most apical part of the junctional complex between the apical and the basolateral cell membrane (Forster 2008). Tight junctions consist of several transmembrane proteins that span the intercellular cleft and thereby constitute a physical barrier. The primary components of tight junctions are the claudins and occludins but also junctional adhesion molecules (JAMs), with the latter proposed to be involved in leukocyte cell adhesion (Abbott *et al.* 2010, Weber *et al.* 2007). In addition, there are scaffold proteins such as *zonula occludens* 1 (ZO-1), ZO-2, and ZO-3 clustering the spanning proteins and connecting them to the actin/myosin cytoskeleton (Niessen 2007). Tight junctions not only form intercellular barriers but also restrict membrane trafficking within one cell, thereby separating the apical from the basolateral membrane domain (Forster 2008). Figure 1.1 illustrates the basic composition and arrangement of tight junctions and adherens junctions at the BBB.

1.1.1.2. Neurovascular unit

The BBB constitutes the main physical barrier between the CNS and the bloodstream. Many other cell types and structures, however, are important for maintaining and regulating the BBB and together they are referred to as the neurovascular unit (Hawkins *et al.* 2005). Neurons, astrocytes, pericytes, and microglia are recognised as particularly important cells within this network but also

structures such as the basal lamina and endothelial glycocalyx (Abbott *et al.* 2012, Neuwelt *et al.* 2011). Anatomically, the endothelial cell layer is closely associated with pericytes and the end-feet of astrocytes, both increasingly recognised to be involved in regulating BBB induction, maintenance, and permeability (Abbott 2013, Abbott *et al.* 2006, Alvarez *et al.* 2011, Armulik *et al.* 2010). In addition, astrocytes provide a cellular link between the endothelial cell layer and neurons with important cell-signalling properties (Abbott *et al.* 2006). Microglia are resident macrophages derived from erythromyeloid precursor cells and are particularly important for an immune response within the brain (Ginhoux *et al.* 2010, Kierdorf *et al.* 2013). The basal membrane is a structure composed of extracellular matrix proteins such as collagen type IV and laminin and surrounds the endothelial cell layer and pericytes (Hawkins *et al.* 2005, Neuwelt *et al.* 2011). The term glycocalyx refers to the collective extracellular sugar layer derived from endothelial cell-membrane glycoproteins. It seems to be involved in regulation of membrane access, for example for blood-circulating leukocytes (Abbott 2013, Haqqani *et al.* 2011).

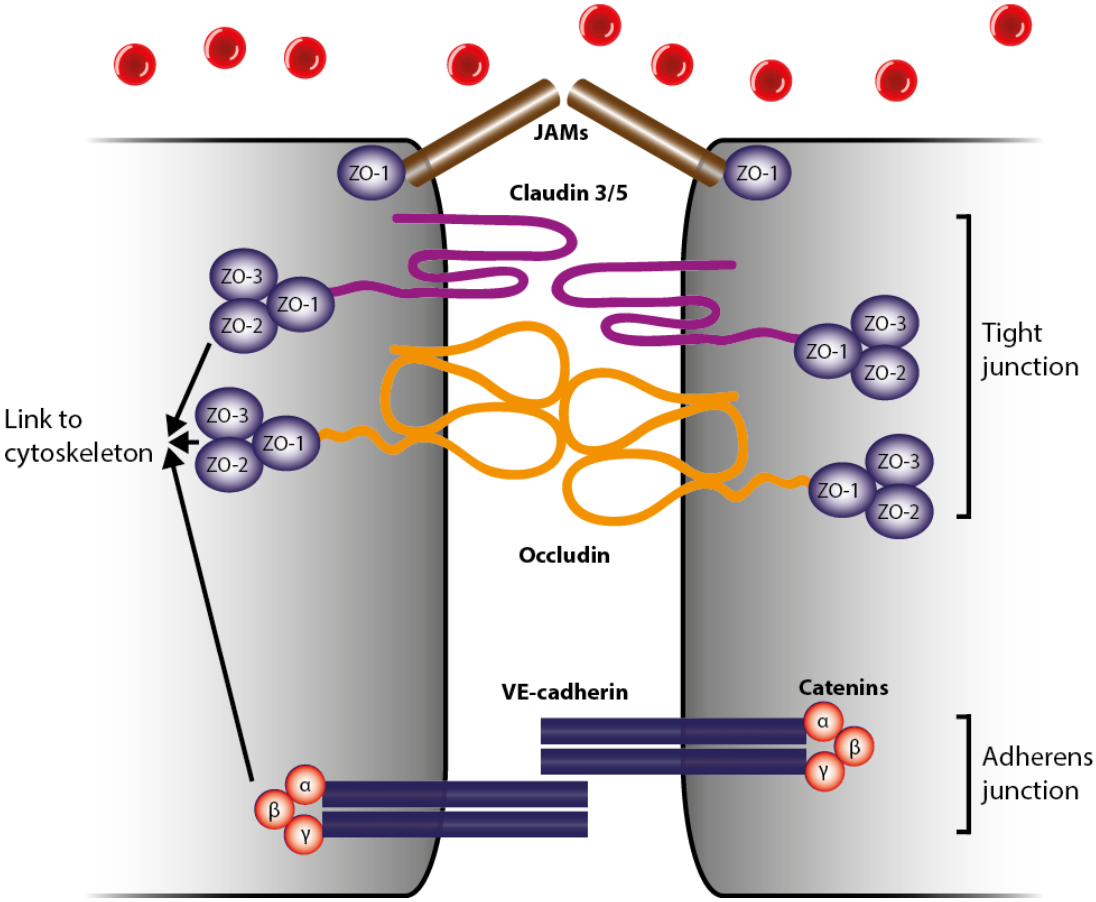


Figure 1.1: Model of blood-brain barrier tight junctions and adherens junctions

Simplified model illustrating tight junction and adherens junction composition and arrangement. Junctional adhesion molecules (JAMs), claudin 3/5, and occludin are important tight junction proteins with the claudins and occludins spanning and "sealing" the intercellular cleft between brain endothelial cells. *Zonula occludens* (ZO) scaffolding proteins cluster and connect tight junction proteins to the actin/myosin cytoskeleton. VE-cadherin proteins are adhesive junction proteins important for structural integrity and tight junction formation. They are linked to the cytoskeleton by means of the catenin scaffolding proteins. Model adapted from Abbott *et al*., 2010

1.1.2. Principles of blood-brain barrier transport

The key feature of the BBB is a tight restriction of paracellular diffusion by means of tight junctions. This makes other mechanisms crucial to allow molecules to pass through this barrier in order to supply the brain with nutrients and remove metabolic waste products. In addition, drug delivery and extrusion is of fundamental interest in pharmacology, particularly in CNS diseases where drugs

usually target receptors in the brain and have to cross the BBB at substantial concentrations (Pardridge 2005). Figure 1.2 illustrates important pathways of BBB drug transit.

The simplest mechanism is the passive transcellular lipophilic pathway (Figure 1.2 A), also referred to as "passive diffusion". Passive diffusion requires a molecule to have certain physicochemical properties to allow entry into the lipid bilayer of the endothelial cells. It has played a decisive role in drug development with regards to CNS drug efficacy and toxicity. The lipophilicity of drugs is generally accepted to be an important determinant for passive diffusion, both *in vitro* and *in vivo*, but correlation becomes poor for highly lipophilic drugs, possibly because of preferential accumulation in the membrane as the surrounding aqueous environment is energetically not favourable for a molecule to exit (Summerfield *et al.* 2007). Other important characteristics for drugs to passively diffuse through the BBB include the molecular size and total hydrogen bond strengths (Sugano *et al.* 2010). Passive diffusion follows a concentration gradient and is usually a non-saturating and non-inhibitable process (Sugano *et al.* 2010).

Figure 1.2 B illustrates the transcytosis pathways, either receptor-mediated or adsorptive-mediated (Abbott *et al.* 2010). Transcytosis pathways, particularly receptor-mediated, have garnered interest in recent years as a drug delivery strategy into the brain. Various macromolecules, such as peptides and proteins, are known to cross the BBB via transcytosis, and biologics, such as monoclonal antibodies, could possibly be modified to overcome BBB restrictions (Watts *et al.* 2013). Finally, the membrane transporter pathway is indicated in Figure 1.2 C+D by the solute carrier (SLC) superfamily and adenosine 5'-triphosphate (ATP)-binding cassette (ABC) family, both of which are described in detail in the next section (1.2).

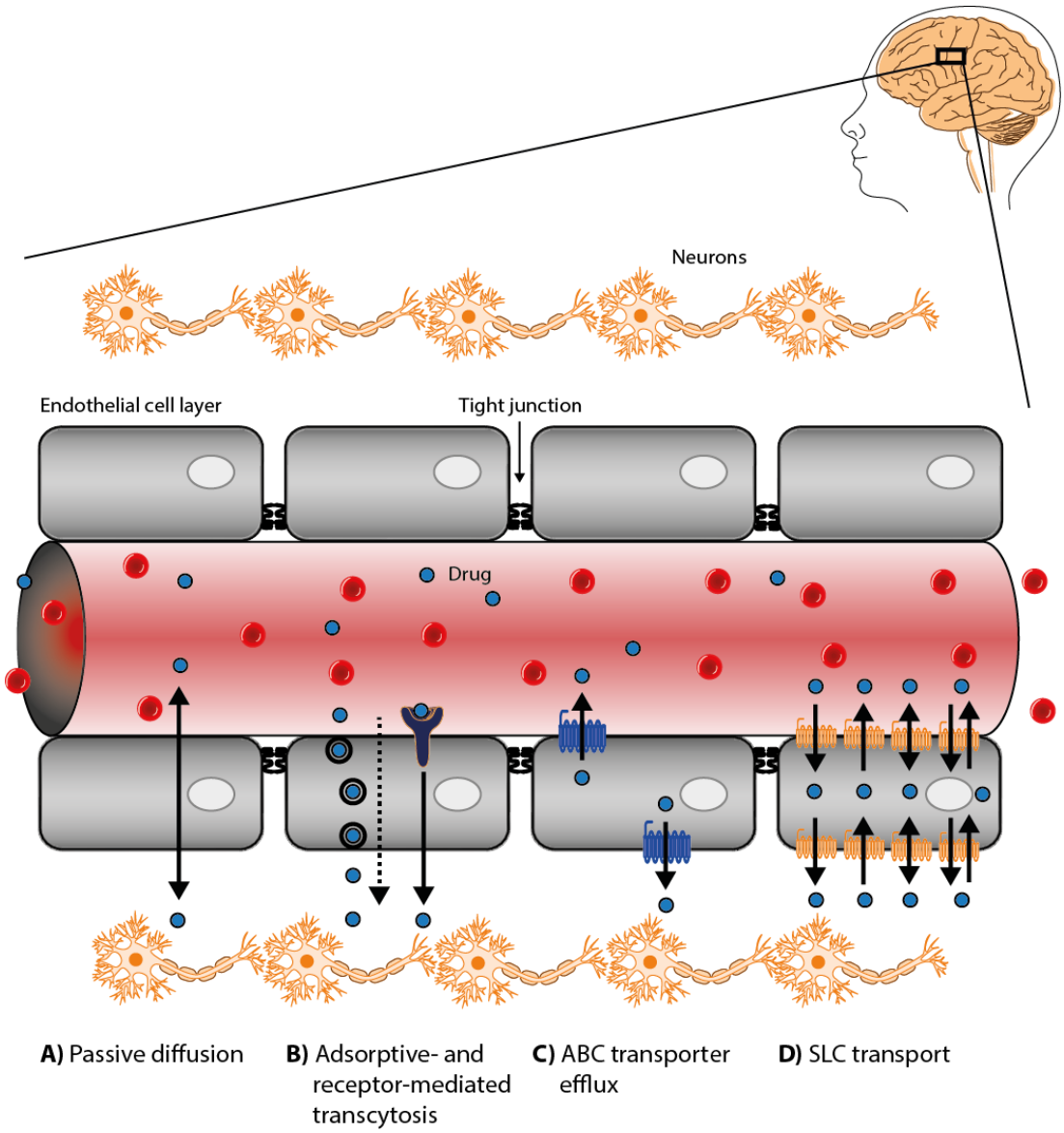


Figure 1.2: Model of blood-brain barrier transport

Simplified model illustrating important drug transport mechanisms across brain endothelial cell layers; A) Passive diffusion; B) Adsorptive-mediated and receptor-mediated transcytosis; C) ABC transporter efflux; D) SLC transport; Model adapted from Abbott *et al*., 2010

1.2. Membrane transporters

Membrane transporters are increasingly recognised as important mediators for drug transport across biological barriers such as the BBB, but also intestinal epithelial cells, hepatocytes, and renal tubule epithelial cells, all of which are characterised by the presence of tight junctions. Each of these barriers expresses a unique and adjustable transportome, owing to the distinct role of the respective organ with specific permeability requirements.

Mechanistically, membrane transporters can be classified as facilitative or active transporters (Hediger *et al.* 2013). Facilitative transporters passively shuttle substrates down their electrochemical concentration gradient with no additional energy source required. Active transporters, in contrast, utilise an energy source to pump substrates against their electrochemical concentration gradient and are classified as primary-active or secondary-active transporters. Primary-active transporters hydrolyse ATP as an energy source whereas secondary-active transporters utilise the electrochemical gradients generated by primary-active transporters in the form of symporters or antiporters (Hediger *et al.* 2013). Primary-active transporters include ion pumps (ATPases) such as the abundantly expressed Na^+/K^+ ATPase (Sandtner *et al.* 2011) and ABC transporters (Hediger *et al.* 2013) (1.2.2). Secondary-active and facilitative transporters often belong to the superfamily of SLC transporters as described in section 1.2.1 (He *et al.* 2009). For example, membrane transporters from the SLC6 family utilise a Na^+ gradient as energy source for substrate translocation (1.2.1.1).

Membrane transporters, such as the SLC superfamily and ABC family, are referred to as carriers and can be functionally distinguished from another class of transport proteins known as channels. The fundamental difference between carriers and channels is that carriers undergo a conformational change to catalyse the translocation of substrates either downhill or uphill of an electrochemical gradient. A carrier is only accessible from one side of the membrane at any given time whereas the pore of a channel may be fully open, allowing substrates to

rapidly diffuse through the pore exclusively down their electrochemical gradient (Gadsby 2009, Shi 2013). Consequently, the turnover rate of a channel can be orders of magnitude higher than that of a carrier (Shi 2013). Two noteworthy types of channels are the water and ion channels with the latter being particularly important in neuronal signalling and acting as targets for antiepileptic drugs (1.3.3). The mechanistic differences between carrier-mediated and channel-mediated transport is illustrated in Figure 1.3.

SLC (1.2.1) and ABC (1.2.2) transporters are playing an emerging role in the field of pharmacokinetics due to the increasing number of drugs known to interact with members of these families. Absorption, distribution, metabolism, and excretion (ADME) are key variables that determine the efficacy or toxicity of drugs. Drug transporters are increasingly recognised as critical components within this network. As for other proteins involved in ADME, drug transporters have the potential to account for critical drug interactions if the transport efficiency of a drug is affected by another compound. In 2010, the International Transporter Consortium (ITC) published a white paper with recommendations for important drug transporters to be considered in drug development and a list of additional emerging transporters was released in 2013 (Giacomini *et al.* 2013, Giacomini *et al.* 2010). In 2012, the United States (US) Food and Drug Administration (FDA) issued a draft industry guideline for drug interaction studies in drug development that included transporters based on the 2010 white paper recommendations (United States Food and Drug Administration 2012). Transporters currently included are ABCB1 (MDR1, Pgp) (1.2.2.1), ABCG2 (BCRP) (1.2.2.3), SLCO1B1 (OATP1B1), SLCO1B3 (OATP1B3) (1.2.1.2), SLC22A2 (OCT2), SLC22A6 (OAT1), and SLC22A8 (OAT3) (1.2.1.3). This list, however, is likely to be expanded soon based on new recommendations from the ITC (Giacomini *et al.* 2013).

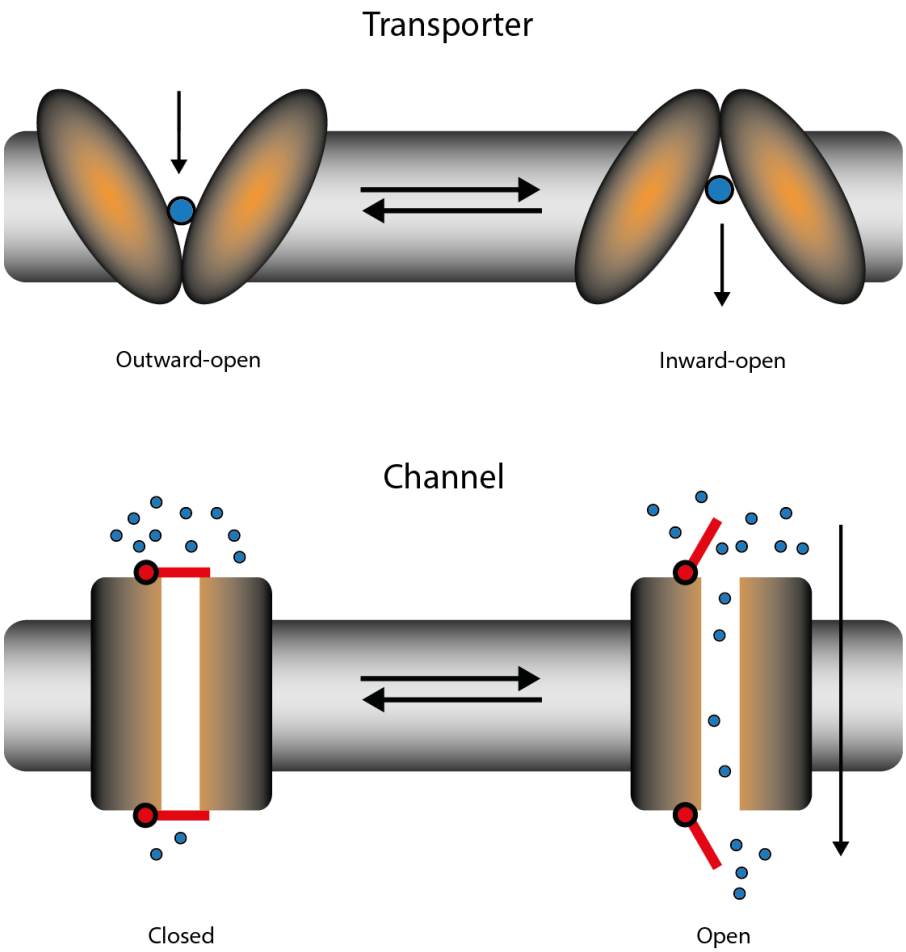


Figure 1.3: Mechanistic model illustrating the transport principles of transporters and channels
Transporters (carriers) catalyse the translocation of substrates either downhill or uphill of an electrochemical gradient. A conformational change of the protein exposes the binding site either to the outside or to the inside. Channels, in contrast, may be open to both sides simultaneously, allowing rapid diffusion of substrates exclusively down their electrochemical gradient. Model adapted from Shi, 2013

1.2.1. Solute carriers

SLCs represent the largest superfamily of membrane transporters and the official list currently consists of 390 *SLC* transporter encoding genes (excluding pseudogenes) within 52 families (*SLC1-SLC52*) and is available as a Human Genome Organisation Gene Nomenclature Committee at the European Bioinformatics Institute (HGNC) approved source at http://slc.bioparadigms.org. A corresponding review article for each family has recently been published in the "Molecular Aspects of Medicine" journal and some selected families are highlighted in more detail in

this section. *SLC* genes are allocated to a family with other genes if the coded proteins share at least 20 % of the amino acid sequence and they may also be further divided into subfamilies (Hediger *et al.* 2013). The official nomenclature for each gene begins with *SLC* to indicate the corresponding gene superfamily and is followed by the family number and subfamily letter. Finally, each gene is allocated a unique number (Hediger *et al.* 2013). The SLC superfamily comprises a diverse set of transporters involved in the absorption and excretion of a broad spectrum of physiologically important endogenous molecules/ions but also xenobiotics such as drugs. For example, members of the SLC2A family are essential for an adequate sugar supply to the brain and other organs (Mueckler *et al.* 2013) while the SLC39A family is essential for metal ion homeostasis, particularly zinc (Jeong *et al.* 2013).

1.2.1.1. SLC6 family

The SLC6 family of membrane transporters currently comprises 20 genes (excluding two putative pseudogenes) and is one of the most clinically relevant groups in the field of neurology. All genes belong to the *SLC6A* subfamily and are further divided into subgroups based on sequence similarities and functions (Kristensen *et al.* 2011). The genes that have gained most interest code for neurotransmitter transporters (NTTs) that play a critical role in neuronal signalling. Among them are inhibitory amino acid neurotransmitter transporters such as the glycine (SLC6A5, SLC6A9) or gamma-aminobutyric acid (GABA, SLC6A1, SLC6A11-13) transporters, and the monoamine neurotransmitter transporters SLC6A2 (NET), SLC6A3 (DAT), and SLC6A4 (SERT), recognising the substrates norepinephrine, dopamine, and serotonin, respectively (Pramod *et al.* 2013).

SLC6 transporters are secondary-active symporters utilising a Na^+ gradient as energy source. In addition, some of these transporters co-transport Cl^- (Pramod *et al.* 2013). The principal function of NTTs is the re-uptake of neurotransmitters from the synaptic cleft into the pre-synapsis, in order to terminate synaptic signal transmission and to recycle the neurotransmitters for further stimuli. Neurotransmitter homoeostasis is a critical component for proper neuronal signalling and imbalances are associated with various neurological diseases,

including mood disorders such as depression, but also epilepsy, schizophrenia, and attention deficit hyperactivity disorder. Accordingly, NTTs are of marked interest as targets for pharmacotherapy with more than 30 predominantly inhibitory drugs currently approved for different conditions (Kristensen *et al.* 2011).

NTT inhibition is usually characterised by prolonged neurotransmitter activity at post-synaptic receptors and the majority of these drugs are indicated for the treatment of mood disorders and target the monoamine neurotransmitter transporters SLC6A2, SLC6A3, and SLC6A4. Targeting drugs belong to the classes of tricyclic antidepressants, selective serotonin/norepinephrine/dopamine re-uptake inhibitors, and non-selective re-uptake inhibitors. An exception is tiagabine, which specifically targets the GABA re-uptake transporter SLC6A1 and is indicated for the treatment of epilepsy (Kristensen *et al.* 2011). SLC6A2, SLC6A3, and SLC6A4 are also known to interact with drugs of abuse such as cocaine, amphetamine, methamphetamine, and 3,4-methylenedioxymethamphetamine ("ecstasy") and hence play an important role in addiction (Kristensen *et al.* 2011).

A breakthrough in understanding the structure and function of SLC6 transporters was achieved by resolving the X-ray crystal structure of the prokaryotic leucine transporter (LeuT) in 2005, a homologue to human SLC6 transporters (Yamashita *et al.* 2005). The three-dimensional structure revealed an unknown but common structural motif for many transporters referred to as the 5+5 inverted repeat fold (Pramod *et al.* 2013). It is characterised by a five transmembrane helix (TMH) bundle that is repeated in a similar form following a pseudo two-fold rotation that is not obvious from the amino acid sequence (Yamashita *et al.* 2005). This motif has subsequently been found in many other prokaryotic transporter X-ray crystal structures with "snapshots" of different stages of a common transport mechanism referred to as the alternate access model (Kristensen *et al.* 2011). In this model, the transporter alternates between different conformational states, the outward-facing open state, the outward-facing occluded state, and the inward-facing open state. The outward-facing open state is characterised by a substrate-binding site accessible from the extracellular environment. Upon substrate binding, the transporter undergoes a conformational change that leads to the outward-

facing occluded state and finally the inward-facing open state with the substrate being released (Kristensen *et al.* 2011).

Interestingly, an X-ray crystal structure of LeuT binding to the competitive inhibitor tryptophan revealed a molecular basis for the mechanism of competitive inhibition as seen with many NTT-targeting drugs. In this structure, tryptophan stabilises the open outward-facing confirmation by binding to the substrate-binding site and therefore sterically hinders the transporter to undergo conformational changes (Singh *et al.* 2008). The basic mechanistic principles of substrate transport and drug inhibition derived from LeuT and other prokaryotic transporters with the 5+5 inverted repeat motif have recently been confirmed with the first eukaryotic Slc6a3 (Dat) transporter X-ray crystal structure derived from *Drosophila melanogaster* that is binding to the competitive inhibitor nortriptyline, a tricyclic antidepressant (Penmatsa *et al.* 2013).

1.2.1.2. SLCO superfamily

The *SLCO* superfamily of membrane transporter genes encodes for the organic anion transporting polypeptides (OATPs) and are recognised as being particularly important for drug transport across biological barriers. The *SLCO* genes were formerly assigned to family *SLC21* within the *SLC* gene nomenclature system (1.2.1). About a decade ago, however, a re-classification was introduced and approved by the HGNC due to major name inconsistencies for newly discovered genes/proteins and a cross-species phylogenetic correlation that revealed the presence of a distinct and species-independent gene superfamily (Hagenbuch *et al.* 2004). This superfamily has subsequently been termed *SLCO* to highlight the relationship to *SLC* transporters, but still allows a species-independent sub-classification into families and subfamilies. Usually, genes are assigned to a *SLCO* family and subfamily if they show ≥ 40 % and ≥ 60 % amino acid sequence identity, respectively (Hagenbuch *et al.* 2004).

The first human *SLCO* gene, *SLCO1A2,* was cloned in 1995, based on a previously cloned rat homologue (Jacquemin *et al.* 1994, Kullak-Ublick *et al.* 1995). Currently, 11 human *SLCO* genes are assigned to 6 families, namely *SLCO1A2*,

SLCO1B1, *SLCO1B3*, *SLCO1C1, SLCO2A1*, *SLCO2B1*, *SLCO3A1*, *SLCO4A1*, *SLCO4C1*, *SLCO5A1*, and *SLCO6A1* (Hagenbuch *et al.* 2013). No crystal structure from any SLCO protein (any species) has been published so far but biochemical data from rat Slco1a1 (Oatp1a1), hydrophobicity plots, and homology modelling suggest that SLCOs consist of 12 TMHs (Hagenbuch *et al.* 2013, Roth *et al.* 2011, Wang *et al.* 2008).

As can be seen from the name, SLCO proteins (OATPs) predominantly mediate the transport, particularly uptake, of endogenous and exogenous organic anions (Obaidat *et al.* 2012). While the exact transport mechanism remains unclear, data from several studies indicate a possible anion exchange process and, at least for some SLCOs, the transport of selected substrates was stimulated by a lower pH, possibly coupled to a secondary-active bicarbonate exchange (Hagenbuch *et al.* 2013, Kobayashi *et al.* 2003, Leuthold *et al.* 2009, Satlin *et al.* 1997). In addition, reduced glutathione (GSH) and GSH-conjugates were demonstrated to act as counter-ions for selected transporters and substrates (Hagenbuch *et al.* 2013, Li *et al.* 1998, Li *et al.* 2000).

While the whole *SLCO* gene superfamily seems to be important in the disposition of endogenous and exogenous substrates, most attention has been drawn on the SLCO1B1 (OATP1B1) and SLCO1B3 (OATP1B3) proteins as both were found to be strongly expressed at the basolateral (sinusoidal) membrane of hepatocytes (Abe *et al.* 1999, Abe *et al.* 2001, Hsiang *et al.* 1999, Konig *et al.* 2000a, b). SLCO1B1 and SLCO1B3 mediate the hepatic uptake of various endogenous substrates, including conjugated and unconjugated bile acids, conjugated and unconjugated bilirubin and thyroid hormones, as well as many important drugs (Hagenbuch *et al.* 2013, Roth *et al.* 2011). Among drugs, examples of identified substrates include statins, such as rosuvastatin (Ho *et al.* 2006), human immunodeficiency virus (HIV) protease inhibitors, such as lopinavir (Hartkoorn *et al.* 2010), and the antineoplastic agent methotrexate (Abe *et al.* 2001). Interestingly, a large and prospective genome-wide association study (GWAS) found *SLCO1B1* polymorphisms to be associated with statin-induced myopathy, particularly at higher doses (Link *et al.* 2008).

SLCO1B1 and SLCO1B3 are not expressed at the BBB (Roth *et al.* 2011). Instead, SLCO1A2 (OATP1A2) and SLCO2B1 (OATP2B1) may be of particular importance for the uptake of drugs into the CNS. Both transporters have been shown to be expressed in brain endothelial cells, localised to the apical membrane (Bronger *et al.* 2005, Lee *et al.* 2005), and are known to recognise numerous substrates. Endogenous substrates of SLCO1A2 are similar to SLCO1B1 and SLCO1B3 and include conjugated and unconjugated bile acids, unconjugated bilirubin, and thyroid hormones (Hagenbuch *et al.* 2013, Roth *et al.* 2011). Accordingly, many drugs that have been identified as substrates for SLCO1B1 and/or SLCO1B3 are also known to be transported by SLCO1A2 (Roth *et al.* 2011) including rosuvastatin, lopinavir, and methotrexate (Badagnani *et al.* 2006, Hartkoorn *et al.* 2010, Ho *et al.* 2006). For SLCO2B1 (OATP2B1) the list of currently known substrates is considerably shorter (Hagenbuch *et al.* 2013, Roth *et al.* 2011). However, this seems to be primarily a result of less extensive research carried out so far and does not necessarily imply a less important role for SLCO2B1. Important endogenous substrates identified include the thyroid hormone thyroxine (T4) and the bile acid taurocholate (Leuthold *et al.* 2009, Nozawa *et al.* 2004). Several drugs have also been reported to be substrates of SLCO2B1, particularly from the group of statins such as rosuvastatin (Ho *et al.* 2006, Roth *et al.* 2011). Despite *SLCO1A2*, *SLCO1B1*, *SLCO1B3*, and *SLCO2B1*, the other 7 currently known human *SLCO* transporter genes might also be of importance for drug disposition, but their role is less clear.

1.2.1.3. SLC22 family

The human *SLC22* family of membrane transporter genes is recognised as another particularly important family encoding for transporters involved in drug disposition. The *SLC22* family currently comprises 23 genes, all assigned to subfamily A. Within the *SLC22A* subfamily, 13 proteins have been functionally characterised and were further assigned to three subgroups, based on primary protein structure similarities (Koepsell 2013). Transporters within these groups often have a similar transport mechanism with overlapping, but still distinct, substrate/inhibitor specificities. They are referred to as organic cation transporters (OCTs), organic zwitterion/cation transporters (OCTNs), and organic anion transporters (OATs) (Koepsell 2013). The

first group comprises SLC22A1-3 (OCT1-3), the second group SLC22A4-5 plus SLC22A16 (OCTN1-2 plus OCT6), and the third group SLC22A6-9 and SLC22A11-13 (OAT1-3, OAT7, OAT4, URAT1, OAT10). Table 1.1 summarises these functionally characterised transporters from the SLC22A subfamily with examples for endogenous and exogenous substrates.

All SLC22 proteins have a similar predicted topology of 12 TMHs with intracellular and extracellular large loops (Koepsell 2013). The large extracellular loop contains several glycosylation sites. Results from *in vitro* experiments with rat Slc22a1 (Oct1), human SLC22A2 (OCT2), and human SLC22A6 (OAT1) suggest that these loops mediate the homo-oligomerisation of the respective transporters and are crucial for proper plasma membrane trafficking (Brast *et al.* 2012, Duan *et al.* 2011, Keller *et al.* 2011). The intracellular loop is characterised by several putative phosphorylation sites involved in posttranscriptional transporter regulation (Koepsell 2013, Koepsell *et al.* 2007).

So far, no X-ray crystal structure has been published for any SLC22 transporter, which would be important to understand the exact transport mechanism. However, extensive mutagenesis experiments have been carried out in addition to homology modelling with rat Slc22a1 based on prokaryotic X-ray crystal structures derived from transporters of the same superfamily, particularly *Escherichia coli* lactose permease (LacY) (Koepsell 2013). Results from these studies suggest a similar basic transport mechanism as described for the prokaryotic LeuT and *Drosophila melanogaster* Slc6a3 (Dat) transporter (1.2.1.1) with an outward-facing open confirmation, outward-facing occluded confirmation, and inward-facing open confirmation, also known as the alternate access model (Abramson *et al.* 2003, Popp *et al.* 2005, Volk *et al.* 2009). Interestingly, whereas LeuT and Slc6a3 exhibit a 5+5 inverted repeat structural motif (1.2.1.1), LacY was found to contain a motif with 2x6 transmembrane bundles related to each other by an approximate two-fold symmetry (Abramson *et al.* 2003). The large substrate-binding cavity is located between these domains that were suggested to undergo a relative rotation to switch between the outward-facing and the inward-facing conformation (Abramson *et al.* 2003). Low- and high-affinity substrate and/or inhibitor binding-

sites that may interact by an allosteric or inhibitory mechanism have been identified in the large cavity of rat Slc22a1 and Slc22a2 (Oct2) for selected compounds utilising site-directed mutagenesis (Egenberger *et al.* 2012, Gorboulev *et al.* 2005, Gorboulev *et al.* 1999, Gorbunov *et al.* 2008, Nies *et al.* 2011b, Popp *et al.* 2005, Volk *et al.* 2009). The presence of high- and low-affinity binding sites has also been suggested for human SLC22A1-3 in experiments utilising transfected cell lines and the HIV nucleoside reverse transcriptase inhibitor (NRTI) lamivudine as inhibitor for 1-methyl-4-phenylpyridinium (MPP^+) uptake, an OCT model substrate (Minuesa *et al.* 2009).

Table 1.1: Overview of functionally characterised transporters from the SLC22A subfamily

Functional subgroup	Transporter	Selected endogenous substrates	Selected exogenous substrates	References
1	SLC22A1 (OCT1)	Cyclo(his-pro), PGE_2	Acyclovir, Lamivudine, Lamotrigine, Metformin	(Dickens *et al.* 2012, Jung *et al.* 2008, Kimura *et al.* 2002, Kimura *et al.* 2005a, Minuesa *et al.* 2009, Nies *et al.* 2011a, Takeda *et al.* 2002b, Taubert *et al.* 2007)
	SLC22A2 (OCT2)	Cyclo(his-pro), Dopamine, Norepinephrine, PGE_2, Serotonin	Lamivudine, Metformin	(Amphoux *et al.* 2006, Busch *et al.* 1998, Jung *et al.* 2008, Kimura *et al.* 2002, Kimura *et al.* 2005a, Kimura *et al.* 2005b, Minuesa *et al.* 2009, Nies *et al.* 2011a, Taubert *et al.* 2007)
	SLC22A3 (OCT3)	Cyclo(his-pro), Norepinephrine, Serotonin	Lamivudine, Lidocaine	(Amphoux *et al.* 2006, Grundemann *et al.* 1998, Hasannejad *et al.* 2004, Minuesa *et al.* 2009, Nies *et al.* 2011a, Taubert *et al.* 2007)
2	SLC22A4 (OCTN1)	Carnitine	Ergothioneine, Gabapentin, Quinidine,	(Dickens *et al.* 2013a, Grundemann *et al.* 2005, Urban *et al.* 2008, Yabuuchi *et*

Functional subgroup	Transporter	Selected endogenous substrates	Selected exogenous substrates	References
			Verapamil	*al.* 1999)
	SLC22A5 (OCTN2)	Carnitine	Quinidine, Verapamil	(Ohashi *et al.* 1999, Tamai *et al.* 1998)
	SLC22A16 (OCT6)	Carnitine	Bleomycin-A5	(Aouida *et al.* 2010, Enomoto *et al.* 2002b)
3	SLC22A6 (OAT1)	PGE_2	MTX, Olmesartan, Tenofovir, Zidovudine	(Bleasby *et al.* 2005, Kimura *et al.* 2002, Takeda *et al.* 2002a, Takeda *et al.* 2002b, Yamada *et al.* 2007)
	SLC22A7 (OAT2)	E3S, PGE_2	5-FU, Paclitaxel, Zidovudine	(Kimura *et al.* 2002, Kobayashi *et al.* 2005, Takeda *et al.* 2002b)
	SLC22A8 (OAT3)	E3S, PGE_2, Urate	MTX, Rosuvastatin, Olmesartan, Zidovudine	(Burckhardt *et al.* 2011, Cha *et al.* 2001, Kimura *et al.* 2002, Takeda *et al.* 2002a, Takeda *et al.* 2002b, Windass *et al.* 2007, Yamada *et al.* 2007)
	SLC22A9 (OAT7)	DHEAS, E3S	-	(Shin *et al.* 2007)
	SLC22A11 (OAT4)	E3S, PGE_2	MTX, Zidovudine	(Burckhardt *et al.* 2011, Kimura *et al.* 2002, Takeda *et al.* 2002a, Takeda *et al.* 2002b)
	SLC22A12 (URAT1)	Urate	-	(Enomoto *et al.* 2002a)
	SLC22A13 (OAT10)	Urate	-	(Bahn *et al.* 2008)

Cyclo(his-pro) = Histidyl-proline diketopiperazine, DHEAS = Dehydroepiandrostone sulfate, E3S = Estrone-3-sulfate, 5-FU = 5-Fluorouracil, MTX = Methotrexate, PGE_2 = Prostaglandin E_2

SLC22A1 (OCT1), SLC22A2 (OCT2), and SLC22A3 (OCT3) constitute the first subgroup of functionally characterised transporters from the SLC22A family. In 1994, the first member, *Slc22a1*, was cloned from a rat cDNA library by means of functional expression cloning. This was followed by cloning of the human *SLC22A1*

gene in 1997 (Gorboulev *et al.* 1997, Grundemann *et al.* 1994, Zhang *et al.* 1997). Corresponding to their name, OCTs predominantly recognise organic cations or weakly alkaline molecules that are positively charged at physiological pH (Nies *et al.* 2011b). 40 % of all orally administered drugs exhibit these properties (Neuhoff *et al.* 2003) and accordingly, a large number of compounds have been found to interact with SLC22A1-3, particularly as inhibitors, with broadly overlapping but still distinctive specificities (Nies *et al.* 2011b).

The translocation of substrates follows a passive facilitated, bi-directional mechanism down the electrochemical gradient (Nies *et al.* 2011b). The large number of identified inhibitors makes substrates susceptible to many potential drug-drug interactions. Drug library screenings with SLC22A1 and SLC22A2 have revealed some common physicochemical features of potent inhibitors such as a positive net charge and high lipophilicity (Ahlin *et al.* 2008, Kido *et al.* 2011).

On the mRNA level, *SLC22A1* is most abundantly expressed in the liver but also detectable in the intestine, kidney, brain, and other organs (Nies *et al.* 2011b). The protein has been localised to the basolateral (sinusoidal) membrane of hepatocytes (Nies *et al.* 2008), apical membrane of renal tubule epithelial cells (Tzvetkov *et al.* 2009), apical membrane of brain endothelial cells (Lin *et al.* 2010), and apical membrane of enterocytes (Han *et al.* 2013), mediating the uptake of substrates from the blood into liver and brain, and from the lumen of the intestine and kidney into the blood. *SLC22A2* mRNA is most significantly detectable in the kidney but also present in the brain, intestine and other tissues (Nies *et al.* 2011b). The protein is localised in the basolateral membrane of renal tubule epithelial cells (Motohashi *et al.* 2002) and the apical membrane of brain endothelial cells (Lin *et al.* 2010), mediating the uptake of substrates from the blood. *SLC22A3* transcripts are detectable in various tissues including the liver, kidney, brain, and intestine (Nies *et al.* 2011b). As for SLC22A1, the SLC22A3 protein is expressed at the basolateral (sinusoidal) membrane of hepatocytes (Nies *et al.* 2009) and the apical membrane of enterocytes (Muller *et al.* 2005). Protein expression has recently also been shown in isolated human brain microvessels but the subcellular localisation remains to be determined (Geier *et al.* 2013).

SLC22A4 (OCTN1), SLC22A5 (OCTN2), and SLC22A16 (OCT6) represent the second subgroup of functionally characterised SLC22A transporters. The first members, *SLC22A4* and *SLC22A5*, were cloned shortly after *SLC22A1* in 1997/1998 and the proteins named OCTN1/OCTN2 due to a unique nucleotide binding sequence motif that is not present in the other OCTs (Tamai *et al.* 1998, Tamai *et al.* 1997). Transporters within this group principally mediate the bi-directional membrane translocation of organic cations and zwitterions (Koepsell 2013). For some substrates and transporters, a Na^+-or H^+-dependency suggests a secondary-active ion exchange as the driving-force (Ohashi *et al.* 1999, Tamai *et al.* 1998, Tamai *et al.* 1997).

SLC22A4 and *SLC22A5* are expressed at the mRNA level in brain, intestine, and kidneys, but only *SLC22A5* mRNA was detected in the adult liver (Koepsell 2013, Meier *et al.* 2007, Tamai *et al.* 1998, Tamai *et al.* 1997). *SLC22A16* mRNA was predominantly found in the testis but not detected in brain, liver, kidney, and the small intestine (Enomoto *et al.* 2002b, Gong *et al.* 2002).

SLC22A6-9 and SLC22A11-13 (OAT1-3, OAT7, OAT4, URAT1, OAT10) proteins belong to the third and largest subgroup of membrane transporters from the SLC22A family. They mediate the transport of organic anions in either direction and are particularly important in the first step of renal excretion (Rizwan *et al.* 2007). Most, if not all, are secondary-active organic anion exchangers utilising counter-ions such as α-ketoglutarate, lactate, and nicotinate (Rizwan *et al.* 2007). So far, all transporters have been functionally characterised (Koepsell 2013), although it remains unclear if any of them is involved in human BBB transport.

SLC22A11 mRNA (coding OAT4) was abundantly present in the kidney but not detectable in brain, liver, and the intestine (Burckhardt *et al.* 2011). A similar expression profile was observed for *SLC22A12* (coding URAT1) but with conflicting data for the brain (Bleasby *et al.* 2006, Burckhardt *et al.* 2011, Nishimura *et al.* 2005). *SLC22A9* (coding OAT7) has been described as exclusively expressed in the liver (Shin *et al.* 2007). The remaining four transporters, *SLC22A6-8* (coding OAT1-3) and *SLC22A13* (coding OAT10), were found to be strongly expressed in the kidney

but mRNA was also detectable in the brain and other organs (Bleasby *et al.* 2006, Burckhardt *et al.* 2011, Nishimura *et al.* 2005). A quantitative absolute proteomics study with isolated human brain microvessels has attempted to analyse the expression of transporters from this group at the human BBB (Uchida *et al.* 2011). None of the transporters was above the limit of quantification. However, the same negative results were obtained for the entire SLC22A family and all 11 human SLCO (OATP) transporters (Uchida *et al.* 2011). Therefore, caution is warranted as there may be issues with the sensitivity of the method. In sharp contrast, recent work that analysed the mRNA expression of most *SLC* transporters in isolated human brain microvessels found the majority of the *SLC22A* genes to be expressed (Geier *et al.* 2013). While involvement at the BBB remains questionable, most transporters from this group are highly expressed in the kidney where they mediate important transport processes. SLC22A6-8 (OAT1-3) have been localised to the basolateral membrane and SLC22A11 (OAT4), SLC22A12 (URAT1), and SLC22A13 (OAT10) to the apical membrane of renal tubule epithelial cells (Burckhardt *et al.* 2011). Together, these transporters constitute a unit that is involved in organic anion transport across the tubule epithelial barrier as the first step in renal excretion (Burckhardt *et al.* 2011).

1.2.1.4. SLC28 and SLC29 families

Genes assigned to families *SLC28* and *SLC29* encode the concentrative nucleoside transporters (CNTs) and the equilibrative nucleoside transporters (ENTs), respectively. *SLC28* consists of three human genes all assigned to subfamily A (*SLC28A1-3*). Similarly, a total of four genes belong to the *SLC29* subfamily *A* (*SLC29A1-4*). *SLC28A1* and *SLC29A1* were the first genes to be cloned and the corresponding proteins functionally characterised from each family (Griffiths *et al.* 1997, Kwong *et al.* 1988, Ritzel *et al.* 1997).

CNTs act as secondary-active symporters and mediate the cellular uptake of substrates against their electrochemical gradients. For SLC28A1 (CNT1) and SLC28A2 (CNT2), a co-transport of Na^+ ions down their electrochemical gradient with a 1:1 stoichiometry has been identified as the driving-force for substrate

translocation (Ritzel *et al.* 1997, Ritzel *et al.* 1998, Smith *et al.* 2004, Smith *et al.* 2007). The driving-force for SLC28A3 (CNT3) substrate translocation is more complex and varies between Na^+ co-transport with a substrate/Na^+ stoichiometry of 1:2 and H^+ co-transport with a substrate/H^+ stoichiometry of 1:1, or may be a mixture (Ritzel *et al.* 2001, Smith *et al.* 2007, Smith *et al.* 2005). Interestingly, SLC28A3 displays different transport characteristics depending on the co-transported cation (Smith *et al.* 2005).

Currently, no X-ray crystal structure is available for any human CNT, but a prokaryotic homologue to SLC28A3 from *Vibrio cholerae* (vcCNT) has recently been published (39 % sequence identity) and the transport mechanism described (Johnson *et al.* 2012). The transporter was found to form homo-trimers with each protomer consisting of eight TMHs, two re-entrant hairpin structures arranged in an opposite direction within the membrane, and three interfacial helices collateral to the membrane (Johnson *et al.* 2012). These components build two subdomains, an outer scaffolding domain that is assumed to be important for the overall structure of the transporter, and an inner transporter domain with conserved amino acid residues that are important for substrate recognition and the transport process. The transport domain contains two related elements arranged in a pseudo two-fold symmetry that is not obvious from the amino acid sequence. The substrate-binding pocket is located in the centre of this symmetric unit in a deep cleft close to the hairpin structures on both sides. A TMH was identified as a critical component that is forming a hydrophobic barrier and blocking substrate access to the intracellular compartment. The authors proposed an alternate access transport mechanism where a “rigid-body motion” moves the substrate binding-pocket across the TMH barrier and exposes it to the opposite side (Johnson *et al.* 2012). Whereas vcCNT contains eight TMHs, the human CNTs have a topology of 12 TMHs as predicated by TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0). Homology modelling with SLC28A2, however, suggested that the human transporters exhibit the same principal structure and transport mechanism as vcCNT and that the additional TMHs are not critically involved in the transport process (Young *et al.* 2013).

Not much is known about the tissue expression of human CNTs. Initially, *SLC28A1* and *SLC28A2* mRNA was detected in the intestine and kidney (Ritzel *et al.* 1998). In the liver, however, only *SLC28A1* mRNA was detectable and no data were presented for the brain (Ritzel *et al.* 1998). *SLC28A3* mRNA expression, in contrast, was studied in more detail and found in different brain regions, the intestine, liver, and kidney (Ritzel *et al.* 2001). Subsequent studies analysing the gene expression of various *SLC* and *ABC* transporters in different human tissues reported conflicting results and a quantitative absolute proteomics study with isolated human brain microvessels failed to find any proteins expressed above the limit of quantification (see 1.2.1.3 for discussion) (Bleasby *et al.* 2006, Nishimura *et al.* 2005, Uchida *et al.* 2011). Immunofluorescence studies reported a predominantly apical membrane localisation for SLC28A1 in enterocytes and renal tubule epithelial cells, while the transporter was mainly expressed at the basolateral (sinusoidal) membrane of hepatocytes (Govindarajan *et al.* 2007, Govindarajan *et al.* 2008). SLC28A2 was expressed on both sides of the membrane in hepatocytes and renal tubule epithelial cells but predominantly in the apical membrane of enterocytes (Govindarajan *et al.* 2007, Govindarajan *et al.* 2008). SLC28A3 was localised in the apical membrane of renal tubule epithelial cells (Damaraju *et al.* 2007). Interestingly, an alternative splice-variant lacking the first N-terminal 69 amino acids (CNT3ins) was found to be retained within the *endoplasmic reticulum* where it showed typical transporter function (Errasti-Murugarren *et al.* 2009).

SLC29A1 (ENT1) and SLC29A2 (ENT2) are passive facilitative transporters and substrates are translocated down their electrochemical gradient in both directions (Young *et al.* 2013). In contrast, SLC29A3 (ENT3) was found to display pH-dependency with a maximum activity at pH 5.5 and no activity at pH 8.0 or higher (Baldwin *et al.* 2005). Correspondingly, SLC29A3 is predominantly localised to intracellular compartments, probably mitochondria and/or lysosomes (Baldwin *et al.* 2005, Govindarajan *et al.* 2009). The last member of this subfamily, SLC29A4 (ENT4), is also known as a pH-dependent and membrane-potential sensitive transporter (Barnes *et al.* 2006, Itagaki *et al.* 2012). In contrast to SLC29A3,

however, SLC29A4 is expressed predominantly at the plasma membrane of cells (Engel *et al.* 2004).

As for the CNTs, no X-ray crystal structure has yet been determined for ENT transporters. In addition, no structure from a homologue prokaryotic transporter is currently available but based on site-directed mutagenesis experiments, structural similarities to LacY (1.2.1.3) have been proposed (Parkinson *et al.* 2011). The predicted number of ENT TMHs is 10-11 as determined by TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0).

On the mRNA level, all *SLC29A* genes are widely expressed in various human tissues, including the brain, liver, kidney, and intestine (Anderson *et al.* 1999, Bleasby *et al.* 2006, Crawford *et al.* 1998, Engel *et al.* 2004, Jennings *et al.* 2001, Nishimura *et al.* 2005). Proteomic analysis of isolated human brain microvessels showed SLC29A1 to be the only protein expressed above the limit of quantification (Uchida *et al.* 2011). Immunofluorescence studies with SLC29A1 and SLC29A2 indicated a predominantly basolateral (sinusoidal membrane) expression in hepatocytes and both sides of the membrane in enterocytes (Govindarajan *et al.* 2007, Govindarajan *et al.* 2008). In addition, SLC29A1 was predominantly detected in either the apical or the basolateral membrane of renal tubule epithelial cells, depending on the exact tubular region (Damaraju *et al.* 2007, Govindarajan *et al.* 2007).

As indicated by the protein names, CNT and ENT transporters mediate the transport of pyrimidine and purine nucleosides across biological barriers with distinct transport and inhibitor characteristics (Parkinson *et al.* 2011). In addition, some ENT transporters, but not CNTs, can also translocate nucleobases (Young *et al.* 2013). Due to their hydrophilic nature, nucleosides and derivatives rely on membrane transporters to cross biological barriers and to enter/exit cells. CNT and ENT transporters are key components in this essential process (Young *et al.* 2013). SLC29A4 is an exception, as this transporter only recognises the nucleoside adenosine at acidic pH (Barnes *et al.* 2006, Engel *et al.* 2004). Instead, SLC29A4 displays similar substrate and inhibitor characteristics as the organic cation

transporters (1.2.1.3) (Engel *et al.* 2005). Corresponding to their physiological function, several CNT and ENT transporters have been characterised as nucleoside drug transporters interacting with many antiviral and anticancer drugs (Parkinson *et al.* 2011). For example, the guanosine analogue ribavirin, indicated for the treatment of hepatitis C, has been identified as a substrate for SLC28A2, SLC28A3, SLC29A1, and SLC29A2 (Yamamoto *et al.* 2007).

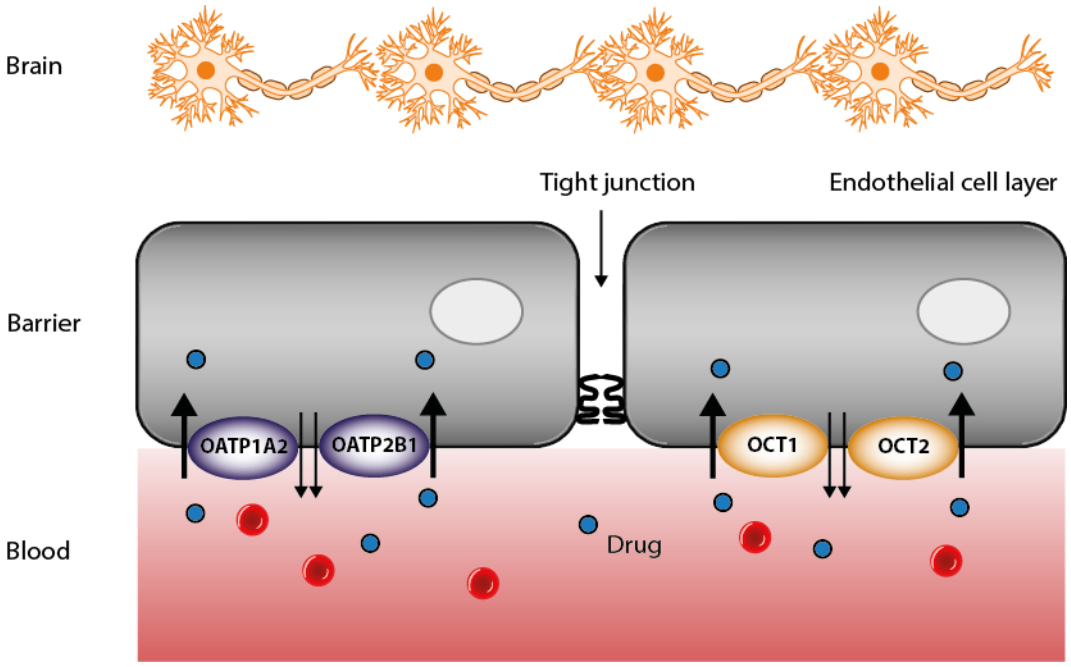


Figure 1.4: Subcellular localisation of selected SLC transporters at the blood-brain barrier

Selected human SLC transporters at the blood-brain barrier as introduced in this thesis chapter with confirmed subcellular localisation

1.2.2. ATP-binding cassette transporters

The family of *ABC* transporter genes currently comprises 48 genes (excluding pseudogenes). The official list is available on the HGNC website (http://www.genenames.org/genefamilies/ABC). The *ABC* family is further divided into seven subfamilies with designated letters *A*-*G* and each gene assigned a unique number following the family root *ABC* and the subfamily letter, e.g. *ABCB1* (1.2.2.1). All ABC transporters, corresponding to their name, are characterised by the presence of two ATP-binding cassettes, also referred to as the nucleotide-binding domains (NBDs). NBDs bind to ATP and the energy derived from ATP hydrolysis is utilised as the driving-force for active substrate translocation against an electrochemical gradient. NBDs typically consist of two highly conserved nucleotide-binding motifs referred to as Walker A and Walker B, linked by another highly conserved motif, the ABC Signature or C motif (Dean *et al.* 2001, Hyde *et al.* 1990, Walker *et al.* 1982). In addition to the NBDs, ABC transporters further comprise two transmembrane domains (TMDs) with varying numbers of TMHs (Deeley *et al.* 2006). While a functional transporter consists of the core structure of two TMDs and two NBDs, the corresponding gene may only encode for a half-transporter with one TMD and one NBD and subsequent homo-dimerisation or hetero-dimerisation at the protein level (Dean *et al.* 2001, Hyde *et al.* 1990).

Functionally, ABC transporters are involved in the unidirectional, active extrusion of xenobiotics and endogenous substances, such as metabolic products and lipids, and thus important cell detoxification systems (Fletcher *et al.* 2010). Along with this function, many ABC transporters are recognised as mediators of a multidrug-resistance phenotype, particularly for cytotoxic anticancer drugs. Many drugs, including anticancer drugs, have been characterised as being substrates for ABC transporters. Some of the most important ABC transporters and examples of substrates will be discussed.

1.2.2.1. ABCB1

The *ABCB1* gene was the first member of the *ABCB* subfamily of efflux transporter genes to be described and is the best-characterised transporter of the entire ABC family. The ABCB1 protein is also referred to as multidrug resistance protein 1 (MDR1) or P-glycoprotein (Pgp). It was initially described in 1976 as a glycoprotein expressed at the surface of drug resistant Chinese hamster ovary cells (Juliano *et al.* 1976). It took another 10 years before the gene was subsequently cloned and the sequence obtained (Chen *et al.* 1986). ABCB1 has been studied for decades and is one of the main drug efflux transporters that is critically involved in the bioavailability of a large number of drugs (Borst *et al.* 2013). This function turns ABCB1 into a double-edged sword with a tissue-protecting effect on the one hand and a mediator for drug resistance on the other, particularly for many highly cytotoxic anticancer agents (Gottesman *et al.* 2002).

In 2009, a high-resolution X-ray crystal structure of mouse Abcb1a was published. It displays 87 % sequence identity with human ABCB1 and confirms the basic structural assumptions that have been made before (Aller *et al.* 2009). Because all ABC transporters have the same basic core structure of two TMDs and two NBDs, the mechanistic insights gained from this crystal structure are of high value for the whole family. Abcb1a was crystallised in the inward-facing confirmation (Figure 1.5) and the two TMDs display a pseudo two-fold symmetry to each other forming a large portal with many hydrophobic amino acid residues localised in the membrane, the assumed substrate binding-pocket (Aller *et al.* 2009). Consistent with the predicted ABCB1 topology, each domain displays a bundle of six TMHs (Aller *et al.* 2009). Mechanistically, the inward-facing structure does not allow substrates to access the binding-pocket from the outer membrane layer or the extracellular space. Instead, it has been suggested that substrates enter the binding-pocket from within the inner membrane layer, which further stimulates ATP binding to the NBDs and is followed by a conformational change of ABCB1 to the outward-facing state (Aller *et al.* 2009). ATP hydrolysis has been suggested to be a likely mechanism to disrupt the dimerisation of both NBDs, and to allow the

transporter to flip back into the inward-facing confirmation (Aller *et al.* 2009, Tombline *et al.* 2005).

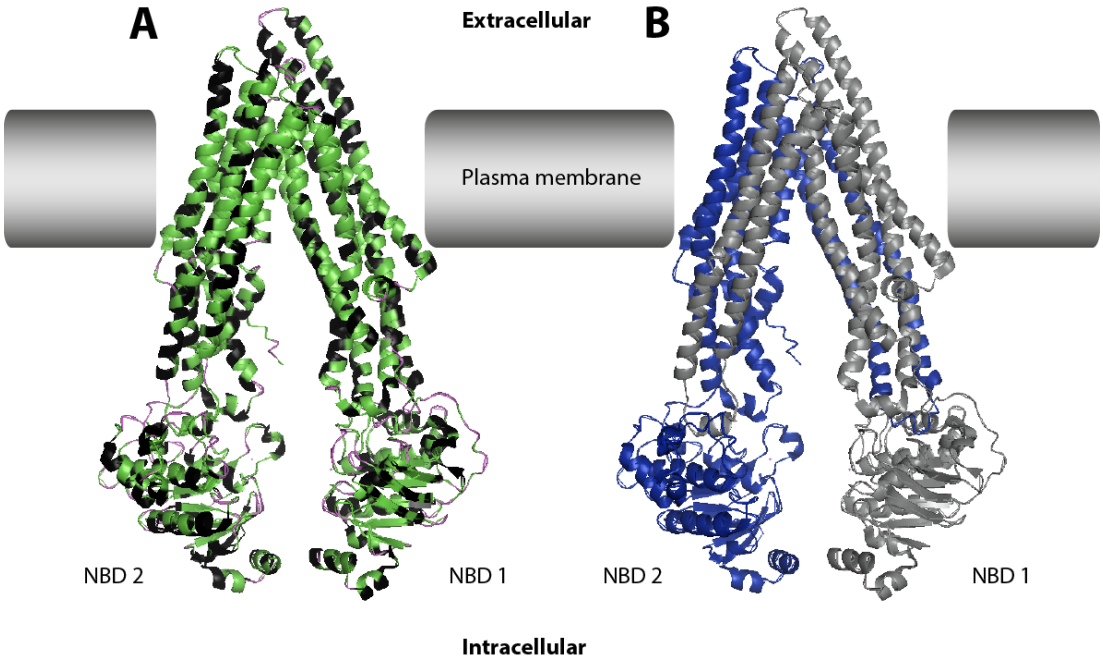


Figure 1.5: Crystal structure of mouse Abcb1a

Cartoon ribbon models derived from the mouse Abcb1a crystal structure in the inward-facing conformation; A) Hydrophobicity model with green indicating hydrophobic amino acid residues; B) Segment model indicating the N-terminal half of the protein (grey) and the C-terminal half (blue), respectively. Each half consists of the typical ABC transporter core structure of one nucleotide-binding domain (NBD) and one transmembrane domain; Models derived from MacPyMOL 1.3 (www.pymol.org) based on Aller *et al*., 2009

ABCB1 is expressed in many human tissues, including barriers such as brain endothelial cells, intestinal epithelial cells, kidney epithelial cells, and hepatocytes, all of which display an apical membrane localisation of the transporter consistent with its role in the efflux/elimination of substrates (Cascorbi 2011). In accordance with the proposed mechanism of ABCB1 transport, ABCB1 substrates are usually hydrophobic molecules (Giacomini *et al.* 2010), and the analysis of a diverse set of known ABCB1 substrates further indicated that substrate-binding is

positively associated with the total hydrogen-bond acceptor strength (Seelig 1998). Examples of ABCB1 substrates include the anticancer drugs doxorubicin and paclitaxel, the cardiac drug digoxin, and the antihistamine fexofenadine (Cascorbi 2011, Giacomini *et al.* 2010).

The *ABCB1* gene is located on chromosome 7. Numerous studies investigated the effect of *ABCB1* genetic variants on drug response. In epilepsy, an association between impaired treatment response to different antiepileptic drugs (AEDs) and the synonymous polymorphism c.3435C>T (rs1045642) was reported (Siddiqui *et al.* 2003). Several subsequent studies, however, failed to confirm these results and its likely role in determining AED efficacy remains controversial (Leschziner *et al.* 2006, Leschziner *et al.* 2007, Sills *et al.* 2005).

1.2.2.2. ABCC subfamily

Twelve genes (excluding one pseudogene) are assigned to the *ABCC* subfamily of membrane transporters designated as *ABCC1-ABCC12*. Based on their functional roles, the encoded proteins are further divided into three classes, namely the multidrug-resistance associated proteins (MRPs), the sulfonylurea receptors (SURs), and the cystic fibrosis transmembrane conductance regulator (CFTR). *ABCC1-ABCC6* and *ABCC10-ABCC12* encode MRP1-9 while *ABCC7* encodes CFTR and *ABCC8-9* the SUR proteins, respectively. MRP transporters, as a general rule, translocate amphiphilic organic anions while CFTR and the SUR proteins are involved in inorganic ion transport and regulation (Bryan *et al.* 2007, Egan *et al.* 1992, Keppler 2011). Depending on the specific substrate and transporter, the transport mechanism of MRPs can be either GSH-independent or GSH-dependent and GSH may either be co-transported with the substrate or it may act as a stimulant (Cole 2013).

Besides the core structure of two TMDs and two NBDs, many ABCC proteins have a predicted third, N-terminal TMD that is possibly involved in protein dimerisation and/or membrane trafficking (Deeley *et al.* 2006, Westlake *et al.* 2005, Yang *et al.* 2010). The predicated ABCC protein core structures and examples for

selected important endogenous and exogenous substrates are summarised in Table 1.2.

Table 1.2: Overview of drug efflux transporters from the ABCC subfamily

Transporter	Predicted third TMD	Selected endogenous substrates	Selected exogenous substrates	References
ABCC1 (MRP1)	Yes	BGB, Bilirubin, $E_2$17βG, LTC_4, MGB	Vincristine	(Jedlitschky *et al.* 1996, Jedlitschky *et al.* 1994, Leier *et al.* 1994, Loe *et al.* 1996, Loe *et al.* 1998, Rigato *et al.* 2004, Westlake *et al.* 2005, Yang *et al.* 2010)
ABCC2 (MRP2)	Yes	BGB, $E_2$17βG, LTC_4, MGB	Cisplatin, Vincristine	(Cui *et al.* 1999, Deeley *et al.* 2006, Kamisako *et al.* 1999, Kawabe *et al.* 1999)
ABCC3 (MRP3)	Yes	BGB, $E_2$17βG, Glycocholate, LTC_4, MGB	MTX	(Deeley *et al.* 2006, Lee *et al.* 2004, Zeng *et al.* 2000)
ABCC4 (MRP4)	No	cAMP, cGMP, Cholate, $E_2$17βG, LTC_4, PGE_1, PGE_2,	MTX, Tenofovir	(Chen *et al.* 2001, Deeley *et al.* 2006, Imaoka *et al.* 2007, Reid *et al.* 2003, Rius *et al.* 2008, Rius *et al.* 2003, van Aubel *et al.* 2002)
ABCC5 (MRP5)	No	cAMP, cGMP	Diglutamylated MTX, MTX, Phosphorylated 5-FU	(Deeley *et al.* 2006, Jedlitschky *et al.* 2000, Pratt *et al.* 2005, Wielinga *et al.* 2005, Wielinga *et al.* 2003)
ABCC6 (MRP6)	Yes	LTC_4, N-ethylmaleimide, S-glutathione	-	(Belinsky *et al.* 2002, Deeley *et al.* 2006, Ilias *et al.* 2002)
ABCC7 (CFTR)	No	Cl^-	-	(Deeley *et al.* 2006, Guggino *et al.* 2006)
ABCC8 (SUR1)	No	-	-	(Bryan *et al.* 2007, Deeley *et al.* 2006)

Transporter	Predicted third TMD	Selected endogenous substrates	Selected exogenous substrates	References
ABCC9 (SUR2)	No	-	-	(Bryan *et al.* 2007, Deeley *et al.* 2006)
ABCC10 (MRP7)	Yes	$E_2$17βG, LTC_4	Paclitaxel, Tenofovir	(Chen *et al.* 2003a, Hopper-Borge *et al.* 2004, Kruh *et al.* 2007, Pushpakom *et al.* 2011)
ABCC11 (MRP8)	No	cAMP, cGMP, Cholylglycine, $E_2$17βG, LTC_4	MTX, Phosphorylated 5-FU	(Chen *et al.* 2005, Guo *et al.* 2003, Kruh *et al.* 2007, Oguri *et al.* 2007)
ABCC12 (MRP9)	No	-	-	(Keppler 2011, Kruh *et al.* 2007)

BGB = Bisglucuronosyl bilirubin, cAMP = Cyclic adenosine monophosphate, cGMP = Cyclic guanosine monophosphate, $E_2$17βG = Estradiol-17β-D-glucuronide, 5-FU = 5-Fluorouracil, LTC_4 = Leukotriene C_4, MGB = Monoglucuronosyl bilirubin, MTX = Methotrexate

ABCC1 was the first human gene cloned and functionally characterised from the *ABCC* subfamily in 1992 and 1994, respectively (Cole *et al.* 1994, Jedlitschky *et al.* 1994, Leier *et al.* 1994). In the initial study, *ABCC1* mRNA was detectable in several human tissues including the lung and testis but not the brain, kidneys, and liver (Cole *et al.* 1992). Subsequent studies, however, reported different results and detected *ABCC1* mRNA or the corresponding protein in many human tissues including the brain, kidneys, liver, and intestine (Bleasby *et al.* 2006, Flens *et al.* 1996, Nies *et al.* 2004, Nishimura *et al.* 2005). Proteomic analysis of isolated human brain microvessels (see 1.2.1.3) failed to detect the protein above the limit of quantification, but an immunolocalisation study reported weak BBB expression in the apical membrane (Nies *et al.* 2004, Uchida *et al.* 2011). In addition, other studies also confirmed mRNA expression in isolated human brain microvessels (Geier *et al.* 2013, Warren *et al.* 2009).

Human and rat *ABCC2* were first cloned in 1996 (Buchler *et al.* 1996, Paulusma *et al.* 1996, Taniguchi *et al.* 1996) following extensive functional work with different rat mutant strains that displayed a hepatocellular apical (canalicular)

transport defect resulting in conjugated hyperbilirubinemia (Jemnitz *et al.* 2010, Nies *et al.* 2007). The first rat strain, referred to as transport-deficient rat strain (TR-), was described in 1985. The authors noted that the phenotype was similar to that seen with Dubin-Johnson syndrome, a disease also characterised by conjugated hyperbilirubinema (Dubin *et al.* 1954, Jansen *et al.* 1985). These observations were later confirmed to be a direct result of mutations in the *ABCC2* gene with impaired ABCC2 (MRP2) transport of conjugated bilirubin from the hepatocytes into the bile (Buchler *et al.* 1996, Paulusma *et al.* 1996, Paulusma *et al.* 1997). ABCC2 is now a well-characterised transporter with a dominant function in the hepatic and renal excretion of conjugated substrates. The expression of *ABCC2* mRNA and ABCC2 protein is particularly strong in liver and kidney, but also detectable in the intestine. In the brain, however, no significant expression has been observed (Bleasby *et al.* 2006, Geier *et al.* 2013, Nies *et al.* 2004, Sandusky *et al.* 2002, Taniguchi *et al.* 1996, Uchida *et al.* 2011, Warren *et al.* 2009). The ABCC2 protein principally displays apical membrane localisation in hepatocytes (canalicular membrane), renal tubule epithelial cells, and enterocytes (Fromm *et al.* 2000, Paulusma *et al.* 1997, Schaub *et al.* 1999). Although membrane localisation and amino acid sequence differs considerably from ABCC1 (MRP1), the substrate specificity seems to be quite similar (see Table 1.2).

ABCC3 gene expression is similar to that of *ABCC2* and mRNA has been detected in liver, kidney, and intestine, but there is only borderline expression in the brain (Bleasby *et al.* 2006, Kiuchi *et al.* 1998, Konig *et al.* 1999, Kool *et al.* 1997, Nies *et al.* 2004). In studies utilising isolated human brain microvessels, conflicting results have been reported with mRNA detectable in two studies but no protein in the quantitative proteomic study (Geier *et al.* 2013, Uchida *et al.* 2011, Warren *et al.* 2009). Membrane localisation of the ABCC3 (MRP3) protein is opposed to that seen with ABCC2 in the basolateral membranes of hepatocytes and renal tubule epithelial cells, respectively (Konig *et al.* 1999, Scheffer *et al.* 2002b). Interestingly, in patients with Dubin-Johnson syndrome hepatic up-regulation of ABCC3 seems to compensate for impaired ABCC2 transport into the bile duct, and might act as a protective mechanism to avoid liver toxicity (Konig *et al.* 1999). Because ABCC3 is

localised to the basolateral rather than the apical membrane, ABCC3-mediated hepatic elimination of bilirubin conjugates is facilitated into the blood and provides an explanation for the observed phenotype. ABCC3 has also been suggested to play an important role in bile acid transport, particularly in the intestine and liver (Deeley *et al.* 2006).

Tissue expression of *ABCC4* mRNA has consistently been reported to be relatively strong in the intestine but conflicting results have been obtained for the liver, kidneys, and brain. Expression in these tissues was either relatively low or not detectable, depending on the study (Bleasby *et al.* 2006, Kool *et al.* 1997, Lee *et al.* 1998, Nies *et al.* 2004, Nishimura *et al.* 2005). Immunolocalisation studies, however, revealed basolateral membrane localisation in hepatocytes (sinusoidal membrane), dominant apical expression in the colon-derived tumour cell lines HT29-CL19A and T84, and an apical expression in brain endothelial cells and renal tubule epithelial cells (Li *et al.* 2007, Nies *et al.* 2004, Rius *et al.* 2003, van Aubel *et al.* 2002). While most ABC and SLC transporters were below the limit of quantification, ABCC4 (MRP4) was one of the few proteins found to be expressed in isolated human brain microvessels (Uchida *et al.* 2011). In agreement with these results, *ABCC4* mRNA was also detectable in two other studies utilising isolated human brain microvessels (Geier *et al.* 2013, Warren *et al.* 2009). ABCC4 displays overlapping but also quite distinct transport characteristics within the ABCC subfamily. It is particularly involved in the translocation of cyclic nucleotides, prostaglandins, and bile acids.

ABCC5 is widely expressed in many human tissues with high mRNA levels in the brain and rather weak mRNA levels detectable in the liver (Bleasby *et al.* 2006, Kool *et al.* 1997, McAleer *et al.* 1999, Nishimura *et al.* 2005). No protein expression was found in isolated human brain microvessels (Uchida *et al.* 2011), but an immunolocalisation study revealed ABCC5 (MRP5) protein expression on the apical side of brain endothelial cells (Nies *et al.* 2004). In addition, mRNA has been detected in isolated human brain microvessels (Geier *et al.* 2013, Warren *et al.* 2009). The substrate profile appears to have distinct overlaps with ABCC4, particularly with regards to nucleotides (see Table 1.2).

At the mRNA level, *ABCC6* is strongly expressed in liver and kidney but also detectable in the intestine. Brain expression, on the other hand, seems to be absent or very weak with potential expression in neurons (Beck *et al.* 2005, Bleasby *et al.* 2006, Kool *et al.* 1999, Nies *et al.* 2004, Nishimura *et al.* 2005). Immunolocalisation studies found the ABCC6 (MRP6) protein to be expressed in the basolateral membrane of renal tubule epithelial cells and hepatocytes (sinusoidal membrane), but no BBB staining was observed (Nies *et al.* 2004, Scheffer *et al.* 2002a). In studies with isolated human brain microvessels, *ABCC6* mRNA was detectable in two studies while proteomic analysis failed to detect any ABCC6 protein (Geier *et al.* 2013, Uchida *et al.* 2011, Warren *et al.* 2009). Interestingly, mutations in the *ABCC6* gene have been linked to the heritable disease *pseudoxanthoma elasticum,* a condition characterised by defects in connective tissue with clinical manifestations in the cardiovascular system, skin, and eyes (Bergen *et al.* 2000, Ringpfeil *et al.* 2000, Struk *et al.* 2000). The pathophysiological role of ABCC6 is still unclear, but evidence suggests that impaired cellular excretion of nucleoside triphosphates into the circulatory system might be involved in the development of this metabolic disorder (Jansen *et al.* 2013).

ABCC7 (CFTR) is a cAMP-dependent Cl^- channel and regulator of many other transport proteins such as aquaporins (Guggino *et al.* 2006). Mutations in the *ABCC7* gene cause cystic fibrosis, a lethal disease characterised by abnormally thick mucous due to impaired epithelial Cl^- transport (Riordan *et al.* 1989, Rommens *et al.* 1989). ABCC8 and ABCC9 (SUR1 and SUR2) proteins are involved in the regulation of K^+ channels and form hetero-octamers with four channel-forming proteins from the $K_{IR}6.x$ subfamily (Bryan *et al.* 2007). These complexes are important regulators of cell excitability and stabilise the membrane resting-potential (Nichols 2006). Complexes with ABCC8 are particularly recognised as being involved in the release of insulin from pancreatic ß-cells (Bryan *et al.* 2007). Sulfonylureas are a class of antidiabetic drugs that target ABCC8 and thereby stimulate the release of insulin in type 2 diabetes (Proks *et al.* 2002). None of these three ABCC transporters has been linked to drug transport so far. The predicted core structure of two TMDs and two

NBDs, however, is the same as for the other ABCC (MRP) transporters (Deeley *et al.* 2006).

ABCC10 mRNA is widely expressed in human tissues including the brain, kidneys, liver, and intestine, but the protein membrane localisation is unknown in these organs (Bleasby *et al.* 2006, Hopper *et al.* 2001, Nishimura *et al.* 2005). Data on BBB expression utilising isolated human brain microvessels are contradictory (Geier *et al.* 2013, Uchida *et al.* 2011, Warren *et al.* 2009). Interestingly, ABCC10 (MRP7) substrates include the HIV nucleotide analogue reverse transcriptase inhibitor tenofovir (see Table 1.2) and a genetic polymorphism in the *ABCC10* gene has recently been associated with tenofovir-induced kidney tubular dysfunction (Pushpakom *et al.* 2011).

ABCC11 mRNA expression was studied by different groups using a panel of human tissues. The results, however, were partially inconsistent. While liver expression was confirmed in five out of five studies, expression in the brain and intestine was reported in four out of five studies and expression in the kidneys in three out of five studies (Bera *et al.* 2001, Bleasby *et al.* 2006, Martin *et al.* 2010, Nishimura *et al.* 2005, Tammur *et al.* 2001, Yabuuchi *et al.* 2001). BBB protein expression is below the limit of quantification (Uchida *et al.* 2011). Very weak mRNA expression has been reported by a different group (Warren *et al.* 2009). Interestingly, the non-synonymous genetic polymorphism 538G>A (rs17822931) in the *ABCC11* gene seems to determine the human earwax type as either being wet (GG or GA genotype) or dry (AA genotype) (Yoshiura *et al.* 2006). The allele frequency is highly population-specific with the G allele being dominant in Europeans and African Americans and the variant in Asian populations such as the Japanese, Chinese, and Koreans (Yoshiura *et al.* 2006). In addition to earwax type, the same polymorphism has been associated with a lower frequency of axillary osmidrosis in a Japanese study (Nakano *et al.* 2009). A subsequent analytical study confirmed that homozygotic carriers of the variant (AA genotype, dry-earwax) display an altered axillary sweat composition lacking certain amino acid conjugates (Martin *et al.* 2010). The authors speculated that these sweat compounds might be substrates for ABCC11 (MRP8) with impaired transport in the AA genotype. A strong

selection-pressure could have resulted in the dominant AA genotype observed in Asians due to mating advantages for carriers of the AA genotype (Martin *et al.* 2010).

ABCC12 mRNA expression studies have been contradictory, ranging from strong expression to no expression (Bleasby *et al.* 2006, Nishimura *et al.* 2005, Tammur *et al.* 2001, Yabuuchi *et al.* 2001). ABCC12 (MRP9) BBB protein expression is below the limit of quantification in isolated human brain microvessels (Uchida *et al.* 2011), but mRNA has been detected (Geier *et al.* 2013). The transporter has so far not been functionally characterised (Keppler 2011, Kruh *et al.* 2007).

1.2.2.3. ABCG2

ABCG2 is the dominant gene associated with drug efflux and multidrug-resistance in the human *ABCG* subfamily. It was initially cloned by three research groups in parallel from a drug-resistant breast cancer cell line, a drug-resistant colon carcinoma cell line, and placenta (Allikmets *et al.* 1998, Doyle *et al.* 1998, Miyake *et al.* 1999). The protein is also commonly referred to as breast cancer resistance protein (BCRP).

The mRNA expression profile has revealed that *ABCG2* is expressed in many human organs including the brain, liver, intestine and kidneys, although two of the original studies failed to detect mRNA in some of these organs using Northern blotting (Allikmets *et al.* 1998, Bleasby *et al.* 2006, Doyle *et al.* 1998, Huls *et al.* 2008, Nishimura *et al.* 2005). ABCG2 displays the same apical membrane localisation as ABCB1 (1.2.2.1), and immunolocalisation studies have confirmed its presence in brain endothelial cells, renal tubule epithelial cells, hepatocytes (canalicular membrane), and enterocytes (Aronica *et al.* 2005, Cooray *et al.* 2002, Huls *et al.* 2008, Maliepaard *et al.* 2001). ABCG2 has also been detected by proteomic analysis (Uchida *et al.* 2011).

Together with ABCB1, ABCG2 is particularly recognised as mediator for multidrug-resistance in cancer with an overlapping and complementary substrate profile to ABCB1 (Fletcher *et al.* 2010). Unlike ABCB1 and the other ABC

transporters discussed so far, the *ABCG2* gene only encodes for one NBD and one TMD. Protein oligomerisation seems to be crucial to produce a functional transporter (Xu *et al.* 2004). Endogenous substrates of ABCG2 include $E_2$17βG and urate, while examples for exogenous substrates include rosuvastatin, the HIV NRTI lamivudine, and the anticancer drug methotrexate including some polyglutamylated metabolites (Chen *et al.* 2003b, Huang *et al.* 2006, Kim *et al.* 2007, Woodward *et al.* 2009). Interestingly, a non-synonymous genetic polymorphism in the nucleotide-binding domain of the *ABCG2* gene has repeatedly displayed strong associations with elevated serum urate levels, which leads to gout. The variant protein has been shown to abolish urate transport in transfected cells *in vitro* (Dehghan *et al.* 2008, Kottgen *et al.* 2013, Woodward *et al.* 2009).

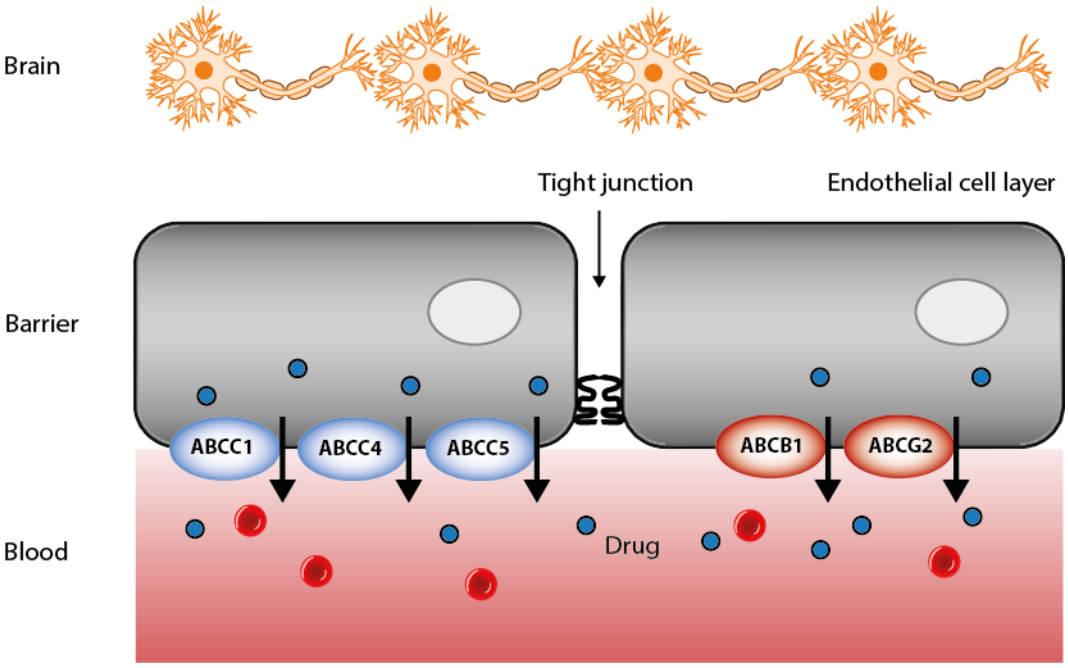


Figure 1.6: Subcellular localisation of selected ABC transporters at the blood-brain barrier

Selected human ABC transporters at the blood-brain barrier as introduced in this thesis chapter with confirmed subcellular localisation

1.3. Epilepsy

1.3.1. General

Epilepsy is a chronic neurological disease affecting around 50 million people worldwide (World Health Organisation 2009). It is characterised by an excessive and synchronised neuronal discharge in the brain that leads to recurrent seizures (Brodie *et al.* 2010, World Health Organisation 2009). Seizures can have a broad range of clinical characteristics and may impair a patient´s consciousness and/or lead to motor, sensory, autonomic, or psychological events (Brodie *et al.* 2010). As a result, seizures can severely interfere with a patient´s quality of life and are often accompanied by secondary events such as injuries. In addition, mortality is increased by a factor of about 2-3 when compared with the general population (Brodie *et al.* 2010).

1.3.2. Classification

Classification of epilepsy is challenging and an on-going matter of discussion. In 2010, the International League Against Epilepsy updated its recommended classification and terminology that is based on proposals from 1981 and 1989, respectively (Berg *et al.* 2010, Commission on Classification and Terminology of the International League Against Epilepsy 1989, 1981). Epilepsy is classified according to a specific electroclinical syndrome in a patient. Electroclinical syndromes consists of groups of clinical features that can be reliably identified by means of diagnostic measurements including the age at seizure onset, underlying genetics, seizure types, and neurological abnormalities (Brodie *et al.* 2010). Epilepsies that cannot be classified by an electroclinical syndrome but still have distinctive clinical characteristics are referred to as constellations. Less distinctive forms of epilepsy are classified as epilepsy secondary to metabolic/structural lesions or as epilepsy of unknown cause (Berg *et al.* 2010).

The most important symptom of epilepsy is a seizure. The type of seizure strongly influences the classification of epilepsy and the prognosis and treatment of

a patient. Seizures are essentially classified by their region of origin and are referred to as generalised seizures or focal seizures. Generalised seizures commonly involve both brain hemispheres and usually impair consciousness. Focal seizures, by contrast, originate locally in one hemisphere and can or cannot involve impaired consciousness. Focal onset seizures can, however, rapidly develop into secondary generalised seizures (Brodie *et al.* 2010). Generalised seizures are further divided into subtypes, namely tonic-clonic, absence, myoclonic, clonic, tonic, and atonic seizures, depending on the exact symptoms (Berg *et al.* 2010). The onset of tonic-clonic seizures is characterised by a sudden contraction of muscles accompanied by loss of consciousness that will cause a patient to fall to the ground. This tonic phase is then followed by the clonic phase with convulsive muscle movements (The Epilepsy Foundation of America 2013).

1.3.3. Pharmacological treatment

Pharmacological treatment is the most important medical strategy to control seizures in epilepsy. The primary goal is to achieve seizure freedom with a minimum of side effects in order to enhance a patient´s quality of life to a level as normal as possible (Brodie *et al.* 2010). A large panel of AEDs are available for treatment, but the exact regimen and dosing requires a careful and patient-specific management. Not all AEDs are indicated for the full seizure spectrum and some can even exacerbate the condition if given for the wrong seizure type (National Institute for Health and Clinical Excellence 2012). In addition to efficacy, adverse reactions and drug interactions are considerably different between AEDs and hence certain drugs are favoured as first-line treatments or in conditions such as pregnancy. Furthermore, cost effectiveness is an increasingly important factor. In general, modern AEDs tend to have a better tolerability when compared to established AEDs (Brodie *et al.* 2010).

Country-specific guidelines are available and recommend how to initiate treatment for different seizure types and how to handle insufficient drug response or adverse reactions. In the UK, patients with epilepsy should be started on appropriate monotherapy, if possible (National Institute for Health and Clinical

Excellence 2012). About 50 % of all patients will achieve remission and tolerate the drug (Brodie *et al.* 2010). The remaining patients should be offered an alternative AED monotherapy before adjunctive treatment is considered due to an increased risk for adverse reactions and drug interactions (National Institute for Health and Clinical Excellence 2012). About 70 % of newly diagnosed patients with epilepsy will achieve full seizure control with AEDs (World Health Organisation 2009) and 30 % will remain refractory (1.3.4).

Three established AEDs, namely carbamazepine (CBZ), valproic acid (VPA), and phenytoin (PHT), plus three modern AEDs, namely lamotrigine (LTG), topiramate (TPM), and levetiracetam (LEV) were studied in this work and their pharmacological properties are described in more detail based on the official FDA-approved prescribing information.

1.3.3.1. Carbamazepine

CBZ is a widely used anticonvulsant drug for the treatment of focal, generalised tonic-clonic, and mixed type seizures. In addition, it is effective in the symptomatic treatment of pain derived from trigeminal neuralgia and may also be used for the treatment of bipolar disorder (Novartis Pharmaceuticals Corporation 2013, Validus Pharmaceuticals LLC 2009). In 1968, CBZ was initially approved by the FDA and still remains an important drug for the treatment of epilepsy. Along with LTG (1.3.3.2) it is recommended as first-line treatment for focal seizures in the UK (National Institute for Health and Clinical Excellence 2012). CBZ seems to exhibit its anticonvulsant activity by blocking voltage-gated sodium channels leading to reduced repetitive firing of neurons (Brodie *et al.* 2010). 76 % of the drug is bound to plasma proteins (Novartis Pharmaceuticals Corporation 2013) and the target serum concentration is between 17-50 μM (Brodie *et al.* 2010).

CBZ undergoes extensive and self-induced metabolism with only 3 % of unchanged parental drug excreted into the urine (Novartis Pharmaceuticals Corporation 2013). The primary route of hepatic CBZ biotransformation in humans is by oxidation of CBZ into the active metabolite CBZ-10,11-epoxide with

subsequent hydrolysis to *trans*-10,11-dihydrodiol-CBZ by microsomal epoxide hydrolase (Frigerio *et al.* 1972, Lertratanangkoon *et al.* 1982, Pirmohamed *et al.* 1992). The major enzyme involved in the first step of this process has been identified as cytochrome P450 (CYP) 3A4 with some minor contributions from CYP2C8 (Kerr *et al.* 1994). Hydroxylated CBZ metabolites undergo further glucuronidation and are finally excreted in the urine (Maggs *et al.* 1997). CBZ is an inducer of its own major metabolising enzyme CYP3A4. Drug therapy is initiated at low and slowly increasing doses (Bertilsson *et al.* 1997, Brodie *et al.* 2010, Pichard *et al.* 1990). About 50 % of all clinically used drugs are known to be substrates of CYP3A4/5 (Bachmann 2009). Therefore, CBZ is at a high risk for clinically relevant drug-drug interactions (Spina *et al.* 1996).

CBZ-induced CYP3A4 seems, at least partly, to be regulated by the nuclear receptor pregnane X (PXR), as first observed in primary human hepatocytes and the hepatocellular carcinoma cell line HepG2 (Lehmann *et al.* 1998, Luo *et al.* 2002). Other nuclear receptors might also be involved, for example CBZ-induced mRNA up-regulation of the constitutive androstane receptor (CAR) has been demonstrated in liver specimens derived from two epilepsy patients (Oscarson *et al.* 2006). Interestingly, CBZ-mediated PXR activation not only seems to induce CYP3A4 but also the expression of drug efflux transporters, particularly ABCB1 (MDR1, Pgp) and ABCC2 (MRP2) (Geick *et al.* 2001, Giessmann *et al.* 2004, Owen *et al.* 2006).

Several other membrane transporters were also differentially expressed at the mRNA level in liver samples derived from patients treated with CBZ as compared to control samples (Oscarson *et al.* 2006). So far, however, direct evidence that CBZ is a substrate for any human ABC transporter, namely ABCB1, ABCC1 (MRP1), ABCC2, ABCC5 (MRP5), and ABCG2 (BCRP), is lacking (Baltes *et al.* 2007b, Cerveny *et al.* 2006, Crowe *et al.* 2006, Dickens *et al.* 2013b, Feng *et al.* 2008, Luna-Tortos *et al.* 2010, 2008, Owen *et al.* 2001, Zhang *et al.* 2011). In addition, SLC transporters have rarely been studied and SLC22A1 (OCT1) is the only transporter where direct CBZ transport has been assessed but with negative outcome (Dickens *et al.* 2012). Consistent with these *in vitro* observations, results from animal studies comparing CBZ brain concentrations in wildtype (WT) and

knockout rodents were also largely negative (Doran *et al.* 2005, Nakanishi *et al.* 2013, Owen *et al.* 2001, Potschka *et al.* 2003, Rizzi *et al.* 2002, Sills *et al.* 2002). To date, results from animal studies utilising knockout models are limited to the Abcb1a, Abcb1b, and Abcg2 transporters in mice and a mutant rat strain (TR-) lacking functional Abcc2 protein expression. Human ABCB1 has two corresponding isoforms in mice, Abcb1a and Abcb1b, and both transporters have been suggested to combine the functions of ABCB1 (Schinkel *et al.* 1996). Based on these studies it seems unlikely that CBZ is a substrate for ABCB1, but there is some evidence that the main CBZ metabolite, CBZ-10,11-epoxide, could be a substrate instead (Schinkel *et al.* 1996, Zhang *et al.* 2011).

The most severe adverse reactions associated with CBZ treatment are serious dermatologic reactions. These reactions can be fatal and include Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). There is growing evidence that these reactions are principally immune-mediated by T-cells (Wu *et al.* 2007, Yip *et al.* 2012). Over the last decade, genetic markers in the human leukocyte antigen (HLA) region, involved in peptide presentation to T-cells, have been associated with severe skin reactions. First, it was demonstrated that Han Chinese patients carrying the HLA-B*1502 allele are at a substantially higher risk for developing SJS and TEN (Chung *et al.* 2004, Hung *et al.* 2006). Later, HLA-A*3101 has been identified to be strongly associated with CBZ-induced hypersensitivity reactions in the Northern European and Japanese populations (Kashiwagi *et al.* 2008, McCormack *et al.* 2011, Ozeki *et al.* 2011). In addition to severe skin reactions, CBZ carries boxed warnings for aplastic anemia and agranulocytosis (Novartis Pharmaceuticals Corporation 2013).

1.3.3.2. Lamotrigine

LTG is a relatively modern antiepileptic drug indicated for the treatment of focal seizures, primary generalised tonic-clonic seizures, and Lennox-Gastaut syndrome. In addition, it is licensed for the maintenance treatment of bipolar I disorder (GlaxoSmithKline Inc. 2012a). LTG received FDA approval in 1994 and, along with CBZ, is the recommended first-line treatment for focal seizures in the UK (National

Institute for Health and Clinical Excellence 2012). The proposed mechanism of action is similar to that of CBZ and involves blocking of voltage-gated sodium channels leading to reduced synaptic release of excitatory neurotransmitters such as glutamate (Brodie *et al.* 2010). Peak plasma concentrations vary between 2-18 μM following single LTG doses of 50 – 400 mg, and about 55 % of the drug is plasma protein bound (GlaxoSmithKline Inc. 2012b).

LTG is metabolised by glucuronic acid conjugation, the main metabolite is the inactive 2-N-glucuronide conjugate catalysed predominantly by uridine 5'-diphospho-glucuronosyltransferase (UGT) isoform 1A4 (Cohen *et al.* 1987, Rowland *et al.* 2006, Sinz *et al.* 1991). No significant interactions with CYP enzymes have been observed, but LTG steady-state concentrations are known to be affected by co-administration of CBZ, VPA, PHT (and other drugs), likely through an induction or inhibition of UGTs (GlaxoSmithKline Inc. 2012a). In addition, LTG is known to self-induce its metabolism when administered as monotherapy (Cohen *et al.* 1987, GlaxoSmithKline Inc. 2012a). About 10 % parental drug and 76 % of the 2-N-glucuronide were found to be eliminated in the urine of healthy volunteers (GlaxoSmithKline Inc. 2012a).

LTG has recently been described as a substrate for human SLC22A1 (Dickens *et al.* 2012) and represents the only SLC transporter studied so far. As with CBZ, most studies investigating direct LTG transport have focused on human ABC transporters. *In vitro* studies assessing the transport by ABCB1 were negative in 3 out of 4 studies (Crowe *et al.* 2006, Dickens *et al.* 2013b, Feng *et al.* 2008, Luna-Tortos *et al.* 2008). In addition, no direct LTG transport was observed for ABCC1, ABCC2, ABCC5, and ABCG2 (Cerveny *et al.* 2006, Luna-Tortos *et al.* 2010). Similarly to these largely negative *in vitro* results, animal studies with WT and knockout mice did not find any support for a role of Abcb1a, Abcb1b, and Abcg2 (Doran *et al.* 2005, Nakanishi *et al.* 2013, Sills *et al.* 2002). In addition, LTG brain concentrations were not affected in Abcc2-deficient TR- rats as compared to controls (Potschka *et al.* 2003).

LTG treatment is associated with potentially severe dermatologic reactions including SJS and TEN. Interestingly, the HLA-A*3101 allele as a predictor for CBZ-induced hypersensitivity reactions in Europeans was not found to be associated with LTG-induced cutaneous adverse drug reactions (McCormack *et al.* 2012).

1.3.3.3. Topiramate

TPM received FDA marketing authorisation in 1996. It is licensed for the treatment of focal seizures, primary generalised tonic-clonic seizures, and seizures that derive from Lennox-Gastaut syndrome. In addition, it may be used as a prophylactic treatment for migraine (Janssen Pharmaceuticals Inc. 2012). Chemically, TPM is notably different to other AEDs and is a sulfamate-substituted, fructose-based monosaccharide (Maryanoff *et al.* 1987, Shank *et al.* 1994). The precise mechanism of action is unknown but involves multiple synaptic targets such as blocking of voltage-gated sodium channels, enhancing the inhibitory effects on a subset of GABA receptors, and antagonising subtypes of the glutamate receptor (Janssen Pharmaceuticals Inc. 2012). TPM peak plasma concentrations ranged from 5 to 85 μM in a singe-dose study with healthy volunteers that received 100 – 1,200 mg TPM (Doose *et al.* 1996). About 15-41 % of the drug is plasma protein bound, depending on the blood concentration (Janssen Pharmaceuticals Inc. 2012).

TPM is not extensively metabolised and about 70 % of unchanged drug is eliminated in the urine (Janssen Pharmaceuticals Inc. 2012). Interestingly, in a study with primary human hepatocytes TPM was found to activate PXR, particularly at higher doses. Consistent with these observations, the expression and activity of CYP3A4 was elevated (Nallani *et al.* 2003). In addition to the mild induction of CYP3A4, TPM is a mild inhibitor of CYP2C19 (Janssen Pharmaceuticals Inc. 2012).

So far, TPM has not been extensively studied as a potential substrate for drug transporters. One *in vitro* study found TPM to be transported by ABCB1 but not ABCC1, ABCC2, and ABCC5 (Luna-Tortos *et al.* 2009). Another study, however, reported negative results for ABCB1 (Crowe *et al.* 2006) and no TPM transport was

observed for SLC22A1 (Dickens *et al.* 2012). Interestingly, data from two studies treating knockout animal models with TPM found increased brain concentrations in *Abcb1a(-/-)* and *Abcb1a/1b(-/-)* mice as compared to WT animals (Nakanishi *et al.* 2013, Sills *et al.* 2002). A triple knockout mouse model, *Abcb1a/Abcb1b/Abcg2(-/-)*, exhibited no further elevated TPM brain to plasma concentration ratio as compared to the double knockout mouse model *Abcb1a/Abcb1b(-/-)* (Nakanishi *et al.* 2013).

TPM treatment has been associated with severe skin reactions including SJS and TEN (Janssen Pharmaceuticals Inc. 2012).

1.3.3.4. Levetiracetam

LEV is a relatively new AED and received FDA marketing authorisation in 1999 for the treatment of focal seizures, primary generalised tonic-clonic seizures, and myoclonic seizures (UCB Inc. 2013). The exact mechanism by which LEV prevents seizures is not fully understood but it seems to be distinctive to other AEDs. *In vitro* studies did not find LEV to block voltage-gates sodium channels or to directly interact with inhibitory GABAergic neurotransmission (UCB Inc. 2013). Instead, data from *in vitro* and animal models demonstrated that LEV binds the synaptic vesicle glycoprotein SV2A (Lynch *et al.* 2004). Although the exact molecular function of SV2A is still unknown, there is some evidence that proteins from the SV2 family could be involved in the regulation of pre-synaptic calcium signalling triggering vesicle exocytosis (Wan *et al.* 2010). LEV peak plasma concentrations reached 182 μM following a 1000 mg single-dose and 253 μM following twice-daily 1000 mg repeated doses (Patsalos 2000).

LEV is not metabolised in the liver and < 10 % is bound to plasma proteins (UCB Inc. 2013). 66 % of the dose is eliminated in the urine as unchanged drug with the major metabolite being a pharmacologically inactive acidic hydrolysis product (24 % of a dose) produced in the blood (Patsalos 2000, UCB Inc. 2013). No clinically relevant interactions with the major drug metabolising enzymes have been observed *in vitro* for both LEV and its main metabolite (UCB Inc. 2013).

Not much information is available about LEV as a potential substrate for drug transporters. Only one research group investigated direct transport by ABCB1, ABCC1, ABCC2, and ABCC5 in two *in vitro* studies and found LEV not to be a substrate for the three ABCC transporters. Conflicting results, however, were obtained for ABCB1 (Baltes *et al.* 2007b, Luna-Tortos *et al.* 2010, 2008). In addition to ABC transporters, LEV is not transported by SLC22A1 (Dickens *et al.* 2012).

Adverse events that are associated with LEV treatment include psychiatric symptoms and serious dermatological reactions such as SJS and TEN (UCB Inc. 2013).

1.3.3.5. Valproic acid

VPA received initial FDA approval in 1978 and is indicated for the treatment of the full seizure spectrum, thereby being particularly important for the treatment of generalised seizures (AbbVie Inc. 2013a, b, Brodie *et al.* 2010). VPA is also indicated for the treatment of manic episodes associated with bipolar disorder and for the prophylaxis of migraine (AbbVie Inc. 2013b). In the UK, VPA is the recommended first-line treatment for newly diagnosed generalised tonic-clonic seizures, absence seizures, myoclonic seizures, tonic seizures, and atonic seizures (National Institute for Health and Clinical Excellence 2012). The precise mechanism of how VPA exerts its antiepileptic properties is unknown but seems to involve facilitated GABAergic neurotransmission by increasing the brain GABA concentrations (AbbVie Inc. 2013a). The target serum concentration is between 350 and 700 μM (Brodie *et al.* 2010).

About 90 % of the drug is plasma protein bound but decreases at higher VPA concentrations (81.5 % at 902 μM) (AbbVie Inc. 2013a). VPA is extensively metabolised in the liver with only 3 % of an administered dose eliminated in the urine. 30-50 % undergoes glucuronidation by various UGT enzymes and another 40 % undergoes mitochondrial ß-oxidation. Oxidation by CYP enzymes only accounts for about 10 % (Ghodke-Puranik *et al.* 2013).

There is some evidence that VPA can induce the expression of *ABCB1* and *CYP3A4* at the mRNA level by means of CAR and/or PXR activation (Cerveny *et al.* 2007). Also, VPA has been demonstrated to induce ABCB1 expression at the protein level in different human tumour cell lines (Eyal *et al.* 2006). Direct evidence for active VPA transport, however, is lacking and results from *in vitro* studies assessing the direct transport by ABCB1, ABCC1, ABCC2, ABCC5, and ABCG2 were negative (Baltes *et al.* 2007a, Cerveny *et al.* 2006, Luna-Tortos *et al.* 2010). In addition, VPA was found not to be transported by SLC22A1 (Dickens *et al.* 2012). Consistent with these observations, no difference in the VPA brain to plasma concentration ratio was observed in triple knockout mice as compared to WT controls for *Abcb1a/Abcb1b/Abcg2(-/-)* animals (Nakanishi *et al.* 2013). Likewise, no difference was observed between WT and Abcc2-deficient TR- rat strains (Baltes *et al.* 2007a).

Potentially life-threatening side effects of VPA include hepatotoxicity and pancreatitis. In addition, VPA is a teratogenic drug that is contraindicated in pregnant women unless medically essential (AbbVie Inc. 2013a).

1.3.3.6. Phenytoin

PHT is one of the oldest AEDs and received initial FDA approval in 1953. It is indicated for the treatment of generalised tonic-clonic and focal seizures as well as for the prevention and the treatment of seizures during/following neurosurgery (Pfizer Inc. 2012). PHT seems to exhibit its antiepileptic properties by inhibiting voltage-gated sodium channels and thereby preventing excessive repetitive firing of neurons (Pfizer Inc. 2012, Thorn *et al.* 2012). The target serum concentration for PHT is between 40-80 μM with 90-93 % of the drug serum protein bound (Brodie *et al.* 2010).

PHT is extensively metabolised in the liver. The main metabolic pathway is the initial oxidation to the pharmacologically inactive hydroxyphenytoin via a reactive arene oxide. This reaction is essentially mediated by CYP2C9 and CYP2C19. Hydroxyphenytoin is subsequently glucuronidated by various UGTs and finally excreted in the urine. (Thorn *et al.* 2012). PHT is a known inducer of various drug

metabolising enzymes and hence susceptible to many drug-drug interactions (Pfizer Inc. 2012). *In vitro* reporter gene assays with human hepatocytes and studies with knockout mice indicated that PHT mediates enzyme induction through activation of CAR but not PXR (Wang *et al.* 2004).

Various groups have undertaken *in vitro* studies to assess the direct transport of PHT by human ABC (1.2.2) transporters, particularly ABCB1 (1.2.2.1). PHT is the only AED that has repeatedly been found to be a weak substrate of ABCB1 by independent groups (Luna-Tortos *et al.* 2008, Schinkel *et al.* 1996, Tishler *et al.* 1995, Zhang *et al.* 2010), but some studies were also negative or inconclusive (Baltes *et al.* 2007b, Crowe *et al.* 2006, Dickens *et al.* 2013b, Feng *et al.* 2008). Results from studies with *ABCC1*, *ABCC2*, *ABCC5* (1.2.2.2), *ABCG2*, and *SLC22A1* transfected cell lines were negative (Baltes *et al.* 2007b, Cerveny *et al.* 2006, Dickens *et al.* 2012, Luna-Tortos *et al.* 2010). Data derived from knockout mouse models also suggest that PHT could be a substrate of Abcb1a/1b but not Abcb1a alone (Doran *et al.* 2005, Nakanishi *et al.* 2013, Rizzi *et al.* 2002, Schinkel *et al.* 1996, Sills *et al.* 2002). In contrast to the *in vitro* results obtained for human ABCC2, one study also reported higher PHT brain concentrations in the Abcc2-deficient TR- rat strain as compared to WT animals, concluding that PHT is a substrate of Abcc2 (Potschka *et al.* 2001).

PHT is a known teratogen and can induce severe hypersensitivity reactions including SJS and TEN (Pfizer Inc. 2012).

1.3.4. Refractory epilepsy

The majority of epilepsy patients achieve proper seizure control when treated with AEDs. However, a substantial number, about 30 %, remain refractory to AED treatment (World Health Organisation 2009). Refractory epilepsy is a major health problem and accompanied by increased morbidity and mortality that severely affects a patient´s quality of life (Devinsky 1999). In an attempt to define epilepsy as pharmacologically refractory, a commission on behalf of The International League

Against Epilepsy suggested that non-response to two tolerated AEDs, either as monotherapy or in combination, can be considered refractory (Kwan *et al.* 2010).

Non-response was defined as not achieving sustained seizure freedom. Mesial temporal lobe epilepsy (MTLE) is a form of epilepsy that is classified as a constellation (1.3.2). It is characterised by a distinctive pathology of hippocampal sclerosis (HS) usually seen by magnetic resonance imaging (MRI). MTLE with HS is one of the commonest causes of refractory epilepsy (Brodie *et al.* 2010). Surgical intervention is a possible alternative for treatment. Superiority to pharmacological treatment has been demonstrated in a randomised controlled study (Wiebe *et al.* 2001) and long-term (5 years) seizure-freedom has been achieved for > 50 % of patients who underwent surgery for MTLE with HS (McIntosh *et al.* 2004).

Brain specimens collected from surgery provide a unique opportunity to study the underlying cause for refractoriness. Several independent research groups found elevated expression of ABC transporters (1.2.2) in these specimens as compared to controls, particularly ABCB1 (MDR1, Pgp) (1.2.2.1) but also ABCC1 (MRP1), ABCC2 (MRP2), and ABCC5 (MRP5) (1.2.2.2) (Aronica *et al.* 2004, Dombrowski *et al.* 2001, Kubota *et al.* 2006, Liu *et al.* 2012, Sisodiya *et al.* 2002, Tishler *et al.* 1995). In addition, a recent *in vivo* positron emission tomography (PET) study confirmed significantly higher transporter activity in refractory patients versus controls utilising (*R*)-[^{11}C]verapamil as a PET tracer and tariquidar as an ABCB1 inhibitor (Feldmann *et al.* 2013). Based on these cumulative findings, it has been hypothesised that enhanced drug efflux at the BBB might contribute to the drug resistant phenotype by lowering the drug concentration in the brain, particularly at the seizure focus. While this is an elegant and plausible explanation, the drug transporter hypothesis would require the majority of all AEDs to be substrates for human drug efflux transporters. Published transporter data, however, have largely been negative or conflicting so far (Cascorbi 2010, Marchi *et al.* 2010).

1.4. Research aims

Membrane transporters are increasingly recognised as important mediators for drug delivery across biological barriers. The concentration of a drug and its potential metabolites is a key factor that will determine a drug's effectiveness and/or toxicity. Membrane transporters can be crucial factors within this system and may affect the concentration of a drug in a particular organ or the whole circulatory system. The CNS is a highly sensitive microenvironment with access restricted by CNS barriers, particularly the BBB. In epilepsy and other neurological diseases, pharmacological targets are usually located within the CNS and drugs therefore have to cross the BBB in order to become effective.

Pharmacological management in epilepsy is ineffective in about 30 % of all patients. The underlying reason, however, remains unknown. A few drug transporters from the ABC family have been reported to be overexpressed in patients with a common form of refractory epilepsy, MTLE with HS. This observation has led to the drug transporter hypothesis, suggesting that enhanced AED efflux at the BBB is leading to inefficient drug delivery to the seizure focus. This hypothesis would, however, require all or most AEDs to be substrates of drug efflux transporters but *in vitro* studies with human ABC transporters have largely been negative or conflicting so far. Table 1.3 summarises the *in vitro* results for six major AEDs from studies that assessed direct transport by human ABC and SLC transporters, respectively. Although these results do not support the transporter hypothesis as an exclusive explanation for refractory epilepsy, drug transport could still be one out of many factors inducing the refractory phenotype for a subset of treatments and/or patients. In addition, the large SLC transporter superfamily has rarely been studied but is important to consider, particularly regarding brain uptake. A concerted effect of various drug influx and efflux transporters with a unique combination for every AED may also be the basis for an extended drug transporter hypothesis. Besides drug efficacy, toxicity and drug-drug interactions are of particular importance and could be a direct result of altered AED transport

processes. Therefore, it is of fundamental importance to understand the transport processes potentially involved in AED ADME.

The aims of this research project were (a) to assess whether CBZ is a substrate of ABCC2 (MRP2) *in vitro* and if previously reported genetic polymorphisms in the *ABCC2* gene contribute to a drug resistant phenotype (b) to identify and characterise new potential transport processes for six major AEDs *in vitro* utilising a BBB model cell line (c) to assess potential clinically relevant drug-drug interactions *in vitro* with LTG as a substrate of SLC22A1 (OCT1).

Table 1.3: Summary of *in vitro* studies that directly assessed the transport of carbamazepine (CBZ), lamotrigine (LTG), topiramate (TPM), levetiracetam (LEV), valproate (VPA), and phenytoin (PHT) by human drug transporters

Drug	Not a substrate	Substrate	References
CBZ	ABCB1		(Owen *et al.* 2001)
	ABCG2		(Cerveny *et al.* 2006)
	ABCB1		(Crowe *et al.* 2006)
	ABCB1, ABCC2		(Baltes *et al.* 2007b)
	ABCB1		(Feng *et al.* 2008)
	ABCB1		(Luna-Tortos *et al.* 2008)
	ABCC1, ABCC2, ABCC5		(Luna-Tortos *et al.* 2010)
	ABCB1		(Zhang *et al.* 2011)
	SLC22A1 (OCT1)		(Dickens *et al.* 2012)
	ABCB1		(Dickens *et al.* 2013b)
LTG	ABCG2		(Cerveny *et al.* 2006)
	ABCB1		(Crowe *et al.* 2006)
	ABCB1		(Feng *et al.* 2008)
		ABCB1	(Luna-Tortos *et al.* 2008)
	ABCC1, ABCC2, ABCC5		(Luna-Tortos *et al.* 2010)
		SLC22A1 (OCT1)	(Dickens *et al.* 2012)
	ABCB1		(Dickens *et al.* 2013b)

Drug	Not a substrate	Substrate	References
TPM	ABCB1		(Crowe *et al.* 2006)
	ABCC1, ABCC2, ABCC5	ABCB1	(Luna-Tortos *et al.* 2009)
	SLC22A1 (OCT1)		(Dickens *et al.* 2012)
LEV	ABCB1, ABCC2		(Baltes *et al.* 2007b)
		ABCB1	(Luna-Tortos *et al.* 2008)
	ABCC1, ABCC2, ABCC5		(Luna-Tortos *et al.* 2010)
	SLC22A1 (OCT1)		(Dickens *et al.* 2012)
VPA	ABCG2		(Cerveny *et al.* 2006)
	ABCB1, ABCC1, ABCC2		(Baltes *et al.* 2007a)
	ABCC1, ABCC2, ABCC5		(Luna-Tortos *et al.* 2010)
	SLC22A1 (OCT1)		(Dickens *et al.* 2012)
PHT		ABCB1	(Tishler *et al.* 1995)
		ABCB1	(Schinkel *et al.* 1996)
	ABCG2		(Cerveny *et al.* 2006)
	ABCB1		(Crowe *et al.* 2006)
	ABCB1, ABCC2		(Baltes *et al.* 2007b)
	ABCB1		(Feng *et al.* 2008)
		ABCB1	(Luna-Tortos *et al.* 2008)
	ABCC1, ABCC2, ABCC5		(Luna-Tortos *et al.* 2010)
		ABCB1	(Zhang *et al.* 2010)
	SLC22A1 (OCT1)		(Dickens *et al.* 2012)
	ABCB1	(ABCB1)*	(Dickens *et al.* 2013b)

* indicates inconclusive results with two positives out of seven test systems

1.5. References

Abbott, N. J. (2013) Blood-brain barrier structure and function and the challenges for CNS drug delivery. *Journal of inherited metabolic disease* **36**, 437-449

Abbott, N. J., and Friedman, A. (2012) Overview and introduction: the blood-brain barrier in health and disease. *Epilepsia* **53 Suppl 6**, 1-6

Abbott, N. J.*, et al.* (2010) Structure and function of the blood-brain barrier. *Neurobiol.Dis.* **37**, 13-25

Abbott, N. J., Ronnback, L., and Hansson, E. (2006) Astrocyte-endothelial interactions at the blood-brain barrier. *Nat.Rev.Neurosci.* **7**, 41-53

AbbVie Inc. (2013a) Depakene Prescribing Information

AbbVie Inc. (2013b) Depakote Prescribing Information

Abe, T.*, et al.* (1999) Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. *The Journal of biological chemistry* **274**, 17159-17163

Abe, T.*, et al.* (2001) LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. *Gastroenterology* **120**, 1689-1699

Abramson, J.*, et al.* (2003) Structure and mechanism of the lactose permease of Escherichia coli. *Science* **301**, 610-615

Ahlin, G.*, et al.* (2008) Structural requirements for drug inhibition of the liver specific human organic cation transport protein 1. *J Med Chem* **51**, 5932-5942

Aller, S. G.*, et al.* (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323**, 1718-1722

Allikmets, R.*, et al.* (1998) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res* **58**, 5337-5339

Alvarez, J. I.*, et al.* (2011) The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Science* **334**, 1727-1731

Amphoux, A.*, et al.* (2006) Differential pharmacological in vitro properties of organic cation transporters and regional distribution in rat brain. *Neuropharmacology* **50**, 941-952

Anderson, C. M.*, et al.* (1999) Distribution of equilibrative, nitrobenzylthioinosine-sensitive nucleoside transporters (ENT1) in brain. *J Neurochem* **73**, 867-873

Aouida, M., Poulin, R., and Ramotar, D. (2010) The human carnitine transporter SLC22A16 mediates high affinity uptake of the anticancer polyamine analogue bleomycin-A5. *The Journal of biological chemistry* **285**, 6275-6284

Armulik, A.*, et al.* (2010) Pericytes regulate the blood-brain barrier. *Nature* **468**, 557-561

Aronica, E.*, et al.* (2004) Expression and cellular distribution of multidrug resistance-related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. *Epilepsia* **45**, 441-451

Aronica, E.*, et al.* (2005) Localization of breast cancer resistance protein (BCRP) in microvessel endothelium of human control and epileptic brain. *Epilepsia* **46**, 849-857

Bachmann, K. (2009) Chapter 8: Drug Metabolism in *Pharmacology* (Hacker, M., Messer, W., and Bachmann, K. eds.), Elsevier Inc. pp 131-173

Badagnani, I.*, et al.* (2006) Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J Pharmacol Exp Ther* **318**, 521-529

Bahn, A.*, et al.* (2008) Identification of a new urate and high affinity nicotinate transporter, hOAT10 (SLC22A13). *The Journal of biological chemistry* **283**, 16332-16341

Baldwin, S. A.*, et al.* (2005) Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. *The Journal of biological chemistry* **280**, 15880-15887

Baltes, S.*, et al.* (2007a) Valproic acid is not a substrate for P-glycoprotein or multidrug resistance proteins 1 and 2 in a number of in vitro and in vivo transport assays. *J.Pharmacol.Exp.Ther.* **320**, 331-343

Baltes, S.*, et al.* (2007b) Differences in the transport of the antiepileptic drugs phenytoin, levetiracetam and carbamazepine by human and mouse P-glycoprotein. *Neuropharmacology* **52**, 333-346

Barnes, K.*, et al.* (2006) Distribution and functional characterization of equilibrative nucleoside transporter-4, a novel cardiac adenosine transporter activated at acidic pH. *Circulation research* **99**, 510-519

Beck, K.*, et al.* (2005) Analysis of ABCC6 (MRP6) in normal human tissues. *Histochemistry and cell biology* **123**, 517-528

Belinsky, M. G.*, et al.* (2002) Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). *Cancer Res* **62**, 6172-6177

Bera, T. K.*, et al.* (2001) MRP8, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. *Molecular medicine (Cambridge, Mass.)* **7**, 509-516

Berg, A. T.*, et al.* (2010) Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. *Epilepsia* **51**, 676-685

Bergen, A. A.*, et al.* (2000) Mutations in ABCC6 cause pseudoxanthoma elasticum. *Nature genetics* **25**, 228-231

Bertilsson, L.*, et al.* (1997) Carbamazepine treatment induces the CYP3A4 catalysed sulphoxidation of omeprazole, but has no or less effect on hydroxylation via CYP2C19. *Br J Clin Pharmacol* **44**, 186-189

Bleasby, K.*, et al.* (2006) Expression profiles of 50 xenobiotic transporter genes in humans and pre-clinical species: a resource for investigations into drug disposition. *Xenobiotica* **36**, 963-988

Bleasby, K.*, et al.* (2005) Functional consequences of single nucleotide polymorphisms in the human organic anion transporter hOAT1 (SLC22A6). *J Pharmacol Exp Ther* **314**, 923-931

Borst, P., and Schinkel, A. H. (2013) P-glycoprotein ABCB1: a major player in drug handling by mammals. *The Journal of clinical investigation* **123**, 4131-4133

Brast, S.*, et al.* (2012) The cysteines of the extracellular loop are crucial for trafficking of human organic cation transporter 2 to the plasma membrane and are involved in oligomerization. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **26**, 976-986

Brodie, M. J., Schachter, S. C., and Kwan, P. (2010) Fast Facts: Epilepsy, *Health Press Limited*

Bronger, H.*, et al.* (2005) ABCC drug efflux pumps and organic anion uptake transporters in human gliomas and the blood-tumor barrier. *Cancer Res* **65**, 11419-11428

Brown, P. D.*, et al.* (2004) Molecular mechanisms of cerebrospinal fluid production. *Neuroscience* **129**, 957-970

Bryan, J.*, et al.* (2007) ABCC8 and ABCC9: ABC transporters that regulate K+ channels. *Pflugers Arch* **453**, 703-718

Buchler, M.*, et al.* (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *The Journal of biological chemistry* **271**, 15091-15098

Burckhardt, G., and Burckhardt, B. C. (2011) In vitro and in vivo evidence of the importance of organic anion transporters (OATs) in drug therapy. *Handb.Exp.Pharmacol.*, 29-104

Busch, A. E.*, et al.* (1998) Human neurons express the polyspecific cation transporter hOCT2, which translocates monoamine neurotransmitters, amantadine, and memantine. *Molecular pharmacology* **54**, 342-352

Cascorbi, I. (2010) ABC transporters in drug-refractory epilepsy: limited clinical significance of pharmacogenetics? *Clinical pharmacology and therapeutics* **87**, 15-18

Cascorbi, I. (2011) P-glycoprotein: Tissue Distribution, Substrates, and Functional Consequences of Genetic Variations. *Handb.Exp.Pharmacol.* **201**, 261-283

Cerveny, L.*, et al.* (2006) Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. *Epilepsia* **47**, 461-468

Cerveny, L.*, et al.* (2007) Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 1032-1041

Cha, S. H.*, et al.* (2001) Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Molecular pharmacology* **59**, 1277-1286

Chen, C. J.*, et al.* (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**, 381-389

Chen, Z. S.*, et al.* (2005) Transport of bile acids, sulfated steroids, estradiol 17-beta-D-glucuronide, and leukotriene C4 by human multidrug resistance protein 8 (ABCC11). *Molecular pharmacology* **67**, 545-557

Chen, Z. S.*, et al.* (2003a) Characterization of the transport properties of human multidrug resistance protein 7 (MRP7, ABCC10). *Molecular pharmacology* **63**, 351-358

Chen, Z. S., Lee, K., and Kruh, G. D. (2001) Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *The Journal of biological chemistry* **276**, 33747-33754

Chen, Z. S.*, et al.* (2003b) Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. *Cancer Res* **63**, 4048-4054

Chung, W. H.*, et al.* (2004) Medical genetics: a marker for Stevens-Johnson syndrome. *Nature* **428**, 486

Cohen, A. F.*, et al.* (1987) Lamotrigine, a new anticonvulsant: pharmacokinetics in normal humans. *Clinical pharmacology and therapeutics* **42**, 535-541

Cole, S. P. (2013) Targeting Multidrug Resistance Protein 1 (MRP1, ABCC1): Past, Present, and Future. *Annu Rev Pharmacol Toxicol*

Cole, S. P.*, et al.* (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258**, 1650-1654

Cole, S. P.*, et al.* (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* **54**, 5902-5910

Commission on Classification and Terminology of the International League Against Epilepsy (1989) Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. *Epilepsia* **30**, 389-399

Commission on Classification and Terminology of the International League Against Epilepsy (1981) Proposal for revised clinical and electroencephalographic classification of epileptic seizures. From the *Epilepsia* **22**, 489-501

Cooray, H. C.*, et al.* (2002) Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport* **13**, 2059-2063

Crawford, C. R.*, et al.* (1998) Cloning of the human equilibrative, nitrobenzylmercaptopurine riboside (NBMPR)-insensitive nucleoside transporter ei by functional expression in a transport-deficient cell line. *The Journal of biological chemistry* **273**, 5288-5293

Crowe, A., and Teoh, Y. K. (2006) Limited P-glycoprotein mediated efflux for anti-epileptic drugs. *J.Drug Target* **14**, 291-300

Cui, Y.*, et al.* (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Molecular pharmacology* **55**, 929-937

Damaraju, V. L.*, et al.* (2007) Localization of broadly selective equilibrative and concentrative nucleoside transporters, hENT1 and hCNT3, in human kidney. *American journal of physiology. Renal physiology* **293**, F200-211

Dean, M., Rzhetsky, A., and Allikmets, R. (2001) The human ATP-binding cassette (ABC) transporter superfamily. *Genome research* **11**, 1156-1166

Deeley, R. G., Westlake, C., and Cole, S. P. (2006) Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiological reviews* **86**, 849-899

Dehghan, A.*, et al.* (2008) Association of three genetic loci with uric acid concentration and risk of gout: a genome-wide association study. *Lancet* **372**, 1953-1961

Devinsky, O. (1999) Patients with refractory seizures. *The New England journal of medicine* **340**, 1565-1570

Dickens, D.*, et al.* (2012) Lamotrigine is a substrate for OCT1 in brain endothelial cells. *Biochemical pharmacology* **83**, 805-814

Dickens, D.*, et al.* (2013a) Transport of gabapentin by LAT1 (SLC7A5). *Biochemical pharmacology* **85**, 1672-1683

Dickens, D.*, et al.* (2013b) A multi-system approach assessing the interaction of anticonvulsants with P-gp. *PloS one* **8**, e64854

Dombrowski, S. M.*, et al.* (2001) Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. *Epilepsia* **42**, 1501-1506

Doose, D. R.*, et al.* (1996) Single-dose pharmacokinetics and effect of food on the bioavailability of topiramate, a novel antiepileptic drug. *J Clin Pharmacol* **36**, 884-891

Doran, A.*, et al.* (2005) The impact of P-glycoprotein on the disposition of drugs targeted for indications of the central nervous system: evaluation using the MDR1A/1B knockout mouse model. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 165-174

Doyle, L. A.*, et al.* (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* **95**, 15665-15670

Duan, P., Li, S., and You, G. (2011) Transmembrane peptide as potent inhibitor of oligomerization and function of human organic anion transporter 1. *Molecular pharmacology* **79**, 569-574

Dubin, I. N., and Johnson, F. B. (1954) Chronic idiopathic jaundice with unidentified pigment in liver cells; a new clinicopathologic entity with a report of 12 cases. *Medicine* **33**, 155-197

Egan, M.*, et al.* (1992) Defective regulation of outwardly rectifying Cl- channels by protein kinase A corrected by insertion of CFTR. *Nature* **358**, 581-584

Egenberger, B.*, et al.* (2012) A substrate binding hinge domain is critical for transport-related structural changes of organic cation transporter 1. *The Journal of biological chemistry* **287**, 31561-31573

Engel, K., and Wang, J. (2005) Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Molecular pharmacology* **68**, 1397-1407

Engel, K., Zhou, M., and Wang, J. (2004) Identification and characterization of a novel monoamine transporter in the human brain. *The Journal of biological chemistry* **279**, 50042-50049

Enomoto, A.*, et al.* (2002a) Molecular identification of a renal urate anion exchanger that regulates blood urate levels. *Nature* **417**, 447-452

Enomoto, A.*, et al.* (2002b) Molecular identification of a novel carnitine transporter specific to human testis. Insights into the mechanism of carnitine recognition. *The Journal of biological chemistry* **277**, 36262-36271

Errasti-Murugarren, E.*, et al.* (2009) A splice variant of the SLC28A3 gene encodes a novel human concentrative nucleoside transporter-3 (hCNT3) protein localized in the endoplasmic reticulum. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 172-182

Eyal, S.*, et al.* (2006) The antiepileptic and anticancer agent, valproic acid, induces P-glycoprotein in human tumour cell lines and in rat liver. *British journal of pharmacology* **149**, 250-260

Farquhar, M. G., and Palade, G. E. (1963) Junctional complexes in various epithelia. *J.Cell Biol.* **17**, 375-412

Feldmann, M.*, et al.* (2013) P-glycoprotein expression and function in patients with temporal lobe epilepsy: a case-control study. *Lancet neurology* **12**, 777-785

Feng, B.*, et al.* (2008) In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. *Drug metabolism and disposition: the biological fate of chemicals* **36**, 268-275

Flens, M. J.*, et al.* (1996) Tissue distribution of the multidrug resistance protein. *The American journal of pathology* **148**, 1237-1247

Fletcher, J. I.*, et al.* (2010) ABC transporters in cancer: more than just drug efflux pumps. *Nat.Rev.Cancer* **10**, 147-156

Forster, C. (2008) Tight junctions and the modulation of barrier function in disease. *Histochem.Cell Biol.* **130**, 55-70

Frigerio, A.*, et al.* (1972) Mass spectrometric characterization of carbamazepine-10,11-epoxide, a carbamazepine metabolite isolated from human urine. *Journal of pharmaceutical sciences* **61**, 1144-1147

Fromm, M. F.*, et al.* (2000) The effect of rifampin treatment on intestinal expression of human MRP transporters. *The American journal of pathology* **157**, 1575-1580

Gadsby, D. C. (2009) Ion channels versus ion pumps: the principal difference, in principle. *Nat Rev Mol Cell Biol* **10**, 344-352

Geick, A., Eichelbaum, M., and Burk, O. (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *The Journal of biological chemistry* **276**, 14581-14587

Geier, E. G.*, et al.* (2013) Profiling solute carrier transporters in the human blood-brain barrier. *Clinical pharmacology and therapeutics* **94**, 636-639

Ghodke-Puranik, Y.*, et al.* (2013) Valproic acid pathway: pharmacokinetics and pharmacodynamics. *Pharmacogenet Genomics* **23**, 236-241

Giacomini, K. M.*, et al.* (2013) International Transporter Consortium commentary on clinically important transporter polymorphisms. *Clinical pharmacology and therapeutics* **94**, 23-26

Giacomini, K. M.*, et al.* (2010) Membrane transporters in drug development. *Nat.Rev.Drug Discov.* **9**, 215-236

Giessmann, T.*, et al.* (2004) Carbamazepine regulates intestinal P-glycoprotein and multidrug resistance protein MRP2 and influences disposition of talinolol in humans. *Clinical pharmacology and therapeutics* **76**, 192-200

Ginhoux, F.*, et al.* (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841-845

GlaxoSmithKline Inc. (2012a) Lamictal Prescribing Information

GlaxoSmithKline Inc. (2012b) Lamictal Product Monograph

Gong, S.*, et al.* (2002) Identification of OCT6 as a novel organic cation transporter preferentially expressed in hematopoietic cells and leukemias. *Experimental hematology* **30**, 1162-1169

Gorboulev, V.*, et al.* (2005) Subtype-specific affinity for corticosterone of rat organic cation transporters rOCT1 and rOCT2 depends on three amino acids within the substrate binding region. *Molecular pharmacology* **67**, 1612-1619

Gorboulev, V.*, et al.* (1997) Cloning and characterization of two human polyspecific organic cation transporters. *DNA and cell biology* **16**, 871-881

Gorboulev, V.*, et al.* (1999) Selectivity of the polyspecific cation transporter rOCT1 is changed by mutation of aspartate 475 to glutamate. *Molecular pharmacology* **56**, 1254-1261

Gorbunov, D.*, et al.* (2008) High-affinity cation binding to organic cation transporter 1 induces movement of helix 11 and blocks transport after mutations in a modeled interaction domain between two helices. *Molecular pharmacology* **73**, 50-61

Gottesman, M. M., Fojo, T., and Bates, S. E. (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat.Rev.Cancer* **2**, 48-58

Govindarajan, R.*, et al.* (2007) In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. *American journal of physiology. Regulatory, integrative and comparative physiology* **293**, R1809-1822

Govindarajan, R.*, et al.* (2008) Expression and hepatobiliary transport characteristics of the concentrative and equilibrative nucleoside transporters in sandwich-cultured human hepatocytes. *American journal of physiology. Gastrointestinal and liver physiology* **295**, G570-580

Govindarajan, R.*, et al.* (2009) Facilitated mitochondrial import of antiviral and anticancer nucleoside drugs by human equilibrative nucleoside transporter-3. *American journal of physiology. Gastrointestinal and liver physiology* **296**, G910-922

Griffiths, M.*, et al.* (1997) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nature medicine* **3**, 89-93

Grundemann, D.*, et al.* (1994) Drug excretion mediated by a new prototype of polyspecific transporter. *Nature* **372**, 549-552

Grundemann, D.*, et al.* (2005) Discovery of the ergothioneine transporter. *Proc Natl Acad Sci U S A* **102**, 5256-5261

Grundemann, D.*, et al.* (1998) Molecular identification of the corticosterone-sensitive extraneuronal catecholamine transporter. *Nature neuroscience* **1**, 349-351

Guggino, W. B., and Stanton, B. A. (2006) New insights into cystic fibrosis: molecular switches that regulate CFTR. *Nat Rev Mol Cell Biol* **7**, 426-436

Guo, Y.*, et al.* (2003) MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. *The Journal of biological chemistry* **278**, 29509-29514

Hagenbuch, B., and Meier, P. J. (2004) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* **447**, 653-665

Hagenbuch, B., and Stieger, B. (2013) The SLCO (former SLC21) superfamily of transporters. *Molecular aspects of medicine* **34**, 396-412

Han, T. K.*, et al.* (2013) Organic Cation Transporter 1 (OCT1/mOct1) is Localized in the Apical Membrane of Caco-2 Cell Monolayers and Enterocytes. *Molecular pharmacology*

Haqqani, A. S.*, et al.* (2011) Methods to study glycoproteins at the blood-brain barrier using mass spectrometry. *Methods in molecular biology (Clifton, N.J.)* **686**, 337-353

Hartkoorn, R. C.*, et al.* (2010) HIV protease inhibitors are substrates for OATP1A2, OATP1B1 and OATP1B3 and lopinavir plasma concentrations are influenced by SLCO1B1 polymorphisms. *Pharmacogenet Genomics* **20**, 112-120

Hasannejad, H.*, et al.* (2004) Human organic cation transporter 3 mediates the transport of antiarrhythmic drugs. *European journal of pharmacology* **499**, 45-51

Hawkins, B. T., and Davis, T. P. (2005) The blood-brain barrier/neurovascular unit in health and disease. *Pharmacological reviews* **57**, 173-185

He, L., Vasiliou, K., and Nebert, D. W. (2009) Analysis and update of the human solute carrier (SLC) gene superfamily. *Hum.Genomics* **3**, 195-206

Hediger, M. A.*, et al.* (2013) The ABCs of membrane transporters in health and disease (SLC series): introduction. *Molecular aspects of medicine* **34**, 95-107

Ho, R. H.*, et al.* (2006) Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. *Gastroenterology* **130**, 1793-1806

Hopper, E.*, et al.* (2001) Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. *Cancer letters* **162**, 181-191

Hopper-Borge, E.*, et al.* (2004) Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. *Cancer Res* **64**, 4927-4930

Hsiang, B.*, et al.* (1999) A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. *The Journal of biological chemistry* **274**, 37161-37168

Huang, L., Wang, Y., and Grimm, S. (2006) ATP-dependent transport of rosuvastatin in membrane vesicles expressing breast cancer resistance protein. *Drug metabolism and disposition: the biological fate of chemicals* **34**, 738-742

Huls, M.*, et al.* (2008) The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. *Kidney international* **73**, 220-225

Hung, S. I.*, et al.* (2006) Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. *Pharmacogenet Genomics* **16**, 297-306

Hyde, S. C.*, et al.* (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* **346**, 362-365

Ilias, A.*, et al.* (2002) Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). *The Journal of biological chemistry* **277**, 16860-16867

Imaoka, T.*, et al.* (2007) Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. *Molecular pharmacology* **71**, 619-627

Itagaki, S.*, et al.* (2012) Electrophysiological characterization of the polyspecific organic cation transporter plasma membrane monoamine transporter. *Drug metabolism and disposition: the biological fate of chemicals* **40**, 1138-1143

Jacquemin, E.*, et al.* (1994) Expression cloning of a rat liver Na(+)-independent organic anion transporter. *Proc Natl Acad Sci U S A* **91**, 133-137

Jansen, P. L., Peters, W. H., and Lamers, W. H. (1985) Hereditary chronic conjugated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. *Hepatology (Baltimore, Md.)* **5**, 573-579

Jansen, R. S.*, et al.* (2013) ABCC6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc Natl Acad Sci U S A*

Janssen Pharmaceuticals Inc. (2012) Topamax Prescribing Information

Jedlitschky, G., Burchell, B., and Keppler, D. (2000) The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *The Journal of biological chemistry* **275**, 30069-30074

Jedlitschky, G.*, et al.* (1996) Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res* **56**, 988-994

Jedlitschky, G.*, et al.* (1994) ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res* **54**, 4833-4836

Jemnitz, K.*, et al.* (2010) ABCC2/Abcc2: a multispecific transporter with dominant excretory functions. *Drug metabolism reviews* **42**, 402-436

Jennings, L. L.*, et al.* (2001) Distinct regional distribution of human equilibrative nucleoside transporter proteins 1 and 2 (hENT1 and hENT2) in the central nervous system. *Neuropharmacology* **40**, 722-731

Jeong, J., and Eide, D. J. (2013) The SLC39 family of zinc transporters. *Molecular aspects of medicine* **34**, 612-619

Johnson, Z. L., Cheong, C. G., and Lee, S. Y. (2012) Crystal structure of a concentrative nucleoside transporter from Vibrio cholerae at 2.4 A. *Nature* **483**, 489-493

Juliano, R. L., and Ling, V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim.Biophys.Acta* **455**, 152-162

Jung, N.*, et al.* (2008) Relevance of the organic cation transporters 1 and 2 for antiretroviral drug therapy in human immunodeficiency virus infection. *Drug metabolism and disposition: the biological fate of chemicals* **36**, 1616-1623

Kamisako, T.*, et al.* (1999) Transport of monoglucuronosyl and bisglucuronosyl bilirubin by recombinant human and rat multidrug resistance protein 2. *Hepatology (Baltimore, Md.)* **30**, 485-490

Kashiwagi, M.*, et al.* (2008) Human leukocyte antigen genotypes in carbamazepine-induced severe cutaneous adverse drug response in Japanese patients. *The Journal of dermatology* **35**, 683-685

Kawabe, T.*, et al.* (1999) Enhanced transport of anticancer agents and leukotriene C4 by the human canalicular multispecific organic anion transporter (cMOAT/MRP2). *FEBS letters* **456**, 327-331

Keller, T.*, et al.* (2011) The large extracellular loop of organic cation transporter 1 influences substrate affinity and is pivotal for oligomerization. *The Journal of biological chemistry* **286**, 37874-37886

Keppler, D. (2011) Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handbook of experimental pharmacology*, 299-323

Kerr, B. M.*, et al.* (1994) Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. *Biochemical pharmacology* **47**, 1969-1979

Kido, Y., Matsson, P., and Giacomini, K. M. (2011) Profiling of a prescription drug library for potential renal drug-drug interactions mediated by the organic cation transporter 2. *J Med Chem* **54**, 4548-4558

Kierdorf, K.*, et al.* (2013) Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nature neuroscience* **16**, 273-280

Kim, H. S.*, et al.* (2007) The effect of ABCG2 V12M, Q141K and Q126X, known functional variants in vitro, on the disposition of lamivudine. *Br J Clin Pharmacol* **64**, 645-654

Kimura, H.*, et al.* (2002) Human organic anion transporters and human organic cation transporters mediate renal transport of prostaglandins. *J Pharmacol Exp Ther* **301**, 293-298

Kimura, N.*, et al.* (2005a) Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1. *Drug Metab Pharmacokinet* **20**, 379-386

Kimura, N., Okuda, M., and Inui, K. (2005b) Metformin transport by renal basolateral organic cation transporter hOCT2. *Pharmaceutical research* **22**, 255-259

Kiuchi, Y.*, et al.* (1998) cDNA cloning and inducible expression of human multidrug resistance associated protein 3 (MRP3). *FEBS letters* **433**, 149-152

Kobayashi, D.*, et al.* (2003) Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J Pharmacol Exp Ther* **306**, 703-708

Kobayashi, Y.*, et al.* (2005) Transport mechanism and substrate specificity of human organic anion transporter 2 (hOat2 [SLC22A7]). *The Journal of pharmacy and pharmacology* **57**, 573-578

Koepsell, H. (2013) The SLC22 family with transporters of organic cations, anions and zwitterions. *Molecular aspects of medicine* **34**, 413-435

Koepsell, H., Lips, K., and Volk, C. (2007) Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharmaceutical research* **24**, 1227-1251

Konig, J.*, et al.* (2000a) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *The Journal of biological chemistry* **275**, 23161-23168

Konig, J.*, et al.* (2000b) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *American journal of physiology. Gastrointestinal and liver physiology* **278**, G156-164

Konig, J.*, et al.* (1999) Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology (Baltimore, Md.)* **29**, 1156-1163

Kool, M.*, et al.* (1997) Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* **57**, 3537-3547

Kool, M.*, et al.* (1999) Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. *Cancer Res* **59**, 175-182

Kottgen, A.*, et al.* (2013) Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. *Nature genetics* **45**, 145-154

Kristensen, A. S.*, et al.* (2011) SLC6 neurotransmitter transporters: structure, function, and regulation. *Pharmacological reviews* **63**, 585-640

Kruh, G. D.*, et al.* (2007) ABCC10, ABCC11, and ABCC12. *Pflugers Arch.* **453**, 675-684

Kubota, H.*, et al.* (2006) Distribution and functional activity of P-glycoprotein and multidrug resistance-associated proteins in human brain microvascular endothelial cells in hippocampal sclerosis. *Epilepsy Res.* **68**, 213-228

Kullak-Ublick, G. A.*, et al.* (1995) Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. *Gastroenterology* **109**, 1274-1282

Kurita, S.*, et al.* (2013) Binding between the junctional proteins afadin and PLEKHA7 and implication in the formation of adherens junction in epithelial cells. *The Journal of biological chemistry* **288**, 29356-29368

Kwan, P.*, et al.* (2010) Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. *Epilepsia* **51**, 1069-1077

Kwong, F. Y.*, et al.* (1988) Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography. *The Biochemical journal* **255**, 243-249

Lee, K.*, et al.* (1998) Isolation of MOAT-B, a widely expressed multidrug resistance-associated protein/canalicular multispecific organic anion transporter-related transporter. *Cancer Res* **58**, 2741-2747

Lee, W.*, et al.* (2005) Polymorphisms in human organic anion-transporting polypeptide 1A2 (OATP1A2): implications for altered drug disposition and central nervous system drug entry. *The Journal of biological chemistry* **280**, 9610-9617

Lee, Y. M.*, et al.* (2004) Identification and functional characterization of the natural variant MRP3-Arg1297His of human multidrug resistance protein 3 (MRP3/ABCC3). *Pharmacogenetics* **14**, 213-223

Lehmann, J. M.*, et al.* (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *The Journal of clinical investigation* **102**, 1016-1023

Leier, I.*, et al.* (1994) The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *The Journal of biological chemistry* **269**, 27807-27810

Lertratanangkoon, K., and Horning, M. G. (1982) Metabolism of carbamazepine. *Drug metabolism and disposition: the biological fate of chemicals* **10**, 1-10

Leschziner, G.*, et al.* (2006) Clinical factors and ABCB1 polymorphisms in prediction of antiepileptic drug response: a prospective cohort study. *Lancet neurology* **5**, 668-676

Leschziner, G. D.*, et al.* (2007) ABCB1 genotype and PGP expression, function and therapeutic drug response: a critical review and recommendations for future research. *Pharmacogenomics.J.* **7**, 154-179

Leuthold, S.*, et al.* (2009) Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. *Am J Physiol Cell Physiol* **296**, C570-582

Li, C.*, et al.* (2007) Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia. *Cell* **131**, 940-951

Li, L.*, et al.* (1998) Identification of glutathione as a driving force and leukotriene C4 as a substrate for oatp1, the hepatic sinusoidal organic solute transporter. *The Journal of biological chemistry* **273**, 16184-16191

Li, L., Meier, P. J., and Ballatori, N. (2000) Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Molecular pharmacology* **58**, 335-340

Lin, C. J.*, et al.* (2010) Cellular localization of the organic cation transporters, OCT1 and OCT2, in brain microvessel endothelial cells and its implication for MPTP transport across the blood-brain barrier and MPTP-induced dopaminergic toxicity in rodents. *J Neurochem* **114**, 717-727

Link, E.*, et al.* (2008) SLCO1B1 variants and statin-induced myopathy--a genomewide study. *The New England journal of medicine* **359**, 789-799

Liu, J. Y.*, et al.* (2012) Neuropathology of the blood-brain barrier and pharmaco-resistance in human epilepsy. *Brain* **135**, 3115-3133

Loe, D. W.*, et al.* (1996) Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *The Journal of biological chemistry* **271**, 9675-9682

Loe, D. W., Deeley, R. G., and Cole, S. P. (1998) Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. *Cancer Res* **58**, 5130-5136

Luna-Tortos, C., Fedrowitz, M., and Loscher, W. (2010) Evaluation of transport of common antiepileptic drugs by human multidrug resistance-associated proteins (MRP1, 2 and 5) that are overexpressed in pharmacoresistant epilepsy. *Neuropharmacology* **58**, 1019-1032

Luna-Tortos, C., Fedrowitz, M., and Loscher, W. (2008) Several major antiepileptic drugs are substrates for human P-glycoprotein. *Neuropharmacology* **55**, 1364-1375

Luna-Tortos, C.*, et al.* (2009) The antiepileptic drug topiramate is a substrate for human P-glycoprotein but not multidrug resistance proteins. *Pharm.Res.* **26**, 2464-2470

Luo, G.*, et al.* (2002) CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 795-804

Lynch, B. A.*, et al.* (2004) The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. *Proc Natl Acad Sci U S A* **101**, 9861-9866

Maggs, J. L.*, et al.* (1997) Characterization of the metabolites of carbamazepine in patient urine by liquid chromatography/mass spectrometry. *Drug metabolism and disposition: the biological fate of chemicals* **25**, 275-280

Maliepaard, M.*, et al.* (2001) Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res* **61**, 3458-3464

Marchi, N.*, et al.* (2010) Transporters in drug-refractory epilepsy: clinical significance. *Clinical pharmacology and therapeutics* **87**, 13-15

Martin, A.*, et al.* (2010) A functional ABCC11 allele is essential in the biochemical formation of human axillary odor. *The Journal of investigative dermatology* **130**, 529-540

Maryanoff, B. E.*, et al.* (1987) Anticonvulsant O-alkyl sulfamates. 2,3:4,5-Bis-O-(1-methylethylidene)-beta-D-fructopyranose sulfamate and related compounds. *J Med Chem* **30**, 880-887

McAleer, M. A.*, et al.* (1999) pABC11 (also known as MOAT-C and MRP5), a member of the ABC family of proteins, has anion transporter activity but does not confer multidrug resistance when overexpressed in human embryonic kidney 293 cells. *The Journal of biological chemistry* **274**, 23541-23548

McCormack, M.*, et al.* (2011) HLA-A*3101 and carbamazepine-induced hypersensitivity reactions in Europeans. *The New England journal of medicine* **364**, 1134-1143

McCormack, M.*, et al.* (2012) Genome-wide mapping for clinically relevant predictors of lamotrigine- and phenytoin-induced hypersensitivity reactions. *Pharmacogenomics* **13**, 399-405

McIntosh, A. M.*, et al.* (2004) Temporal lobectomy: long-term seizure outcome, late recurrence and risks for seizure recurrence. *Brain* **127**, 2018-2030

Meier, Y.*, et al.* (2007) Regional distribution of solute carrier mRNA expression along the human intestinal tract. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 590-594

Minuesa, G.*, et al.* (2009) Transport of lamivudine [(-)-beta-L-2',3'-dideoxy-3'-thiacytidine] and high-affinity interaction of nucleoside reverse transcriptase inhibitors with human organic cation transporters 1, 2, and 3. *J Pharmacol Exp Ther* **329**, 252-261

Miyake, K.*, et al.* (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res* **59**, 8-13

Motohashi, H.*, et al.* (2002) Gene expression levels and immunolocalization of organic ion transporters in the human kidney. *Journal of the American Society of Nephrology : JASN* **13**, 866-874

Mueckler, M., and Thorens, B. (2013) The SLC2 (GLUT) family of membrane transporters. *Molecular aspects of medicine* **34**, 121-138

Muller, J.*, et al.* (2005) Drug specificity and intestinal membrane localization of human organic cation transporters (OCT). *Biochemical pharmacology* **70**, 1851-1860

Nakanishi, H.*, et al.* (2013) Impact of P-glycoprotein and breast cancer resistance protein on the brain distribution of antiepileptic drugs in knockout mouse models. *European journal of pharmacology* **710**, 20-28

Nakano, M.*, et al.* (2009) A strong association of axillary osmidrosis with the wet earwax type determined by genotyping of the ABCC11 gene. *BMC genetics* **10**, 42

Nallani, S. C.*, et al.* (2003) Dose-dependent induction of cytochrome P450 (CYP) 3A4 and activation of pregnane X receptor by topiramate. *Epilepsia* **44**, 1521-1528

National Institute for Health and Clinical Excellence (2012) The epilepsies: the diagnosis and management of the epilepsies in adults and children in primary and secondary care (NICE clinical guideline 137)

Neuhoff, S.*, et al.* (2003) pH-dependent bidirectional transport of weakly basic drugs across Caco-2 monolayers: implications for drug-drug interactions. *Pharmaceutical research* **20**, 1141-1148

Neuwelt, E. A.*, et al.* (2011) Engaging neuroscience to advance translational research in brain barrier biology. *Nature reviews. Neuroscience* **12**, 169-182

Nichols, C. G. (2006) KATP channels as molecular sensors of cellular metabolism. *Nature* **440**, 470-476

Nies, A. T.*, et al.* (2008) Vectorial transport of the plant alkaloid berberine by double-transfected cells expressing the human organic cation transporter 1 (OCT1, SLC22A1) and the efflux pump MDR1 P-glycoprotein (ABCB1). *Naunyn-Schmiedeberg's archives of pharmacology* **376**, 449-461

Nies, A. T.*, et al.* (2011a) Proton pump inhibitors inhibit metformin uptake by organic cation transporters (OCTs). *PloS one* **6**, e22163

Nies, A. T.*, et al.* (2004) Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. *Neuroscience* **129**, 349-360

Nies, A. T., and Keppler, D. (2007) The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch* **453**, 643-659

Nies, A. T.*, et al.* (2011b) Organic Cation Transporters (OCTs, MATEs), In Vitro and In Vivo Evidence for the Importance in Drug Therapy. *Handb.Exp.Pharmacol.* **201**, 105-167

Nies, A. T.*, et al.* (2009) Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology (Baltimore, Md.)* **50**, 1227-1240

Niessen, C. M. (2007) Tight junctions/adherens junctions: basic structure and function. *J.Invest Dermatol.* **127**, 2525-2532

Nishimura, M., and Naito, S. (2005) Tissue-specific mRNA expression profiles of human ATP-binding cassette and solute carrier transporter superfamilies. *Drug Metab Pharmacokinet* **20**, 452-477

Novartis Pharmaceuticals Corporation (2013) Tegretol Medication Guideline

Nozawa, T.*, et al.* (2004) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J Pharmacol Exp Ther* **308**, 438-445

Obaidat, A., Roth, M., and Hagenbuch, B. (2012) The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. *Annu Rev Pharmacol Toxicol* **52**, 135-151

Oguri, T.*, et al.* (2007) MRP8/ABCC11 directly confers resistance to 5-fluorouracil. *Molecular cancer therapeutics* **6**, 122-127

Ohashi, R.*, et al.* (1999) Na(+)-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. *J Pharmacol Exp Ther* **291**, 778-784

Oscarson, M.*, et al.* (2006) Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. *Clinical pharmacology and therapeutics* **80**, 440-456

Owen, A.*, et al.* (2006) Induction of P-glycoprotein in lymphocytes by carbamazepine and rifampicin: the role of nuclear hormone response elements. *Br J Clin Pharmacol* **62**, 237-242

Owen, A.*, et al.* (2001) Carbamazepine is not a substrate for P-glycoprotein. *Br.J.Clin.Pharmacol.* **51**, 345-349

Ozeki, T.*, et al.* (2011) Genome-wide association study identifies HLA-A*3101 allele as a genetic risk factor for carbamazepine-induced cutaneous adverse drug reactions in Japanese population. *Human molecular genetics* **20**, 1034-1041

Pardridge, W. M. (2005) The blood-brain barrier: bottleneck in brain drug development. *NeuroRx.* **2**, 3-14

Parkinson, F. E.*, et al.* (2011) Molecular biology of nucleoside transporters and their distributions and functions in the brain. *Current topics in medicinal chemistry* **11**, 948-972

Patsalos, P. N. (2000) Pharmacokinetic profile of levetiracetam: toward ideal characteristics. *Pharmacology & therapeutics* **85**, 77-85

Paulusma, C. C.*, et al.* (1996) Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271**, 1126-1128

Paulusma, C. C.*, et al.* (1997) A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome. *Hepatology (Baltimore, Md.)* **25**, 1539-1542

Penmatsa, A., Wang, K. H., and Gouaux, E. (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. *Nature* **503**, 85-90

Pfizer Inc. (2012) Dilantin Medication Guide

Pichard, L.*, et al.* (1990) Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug metabolism and disposition: the biological fate of chemicals* **18**, 595-606

Pirmohamed, M.*, et al.* (1992) An investigation of the formation of cytotoxic, protein-reactive and stable metabolites from carbamazepine in vitro. *Biochemical pharmacology* **43**, 1675-1682

Popp, C.*, et al.* (2005) Amino acids critical for substrate affinity of rat organic cation transporter 1 line the substrate binding region in a model derived from the tertiary structure of lactose permease. *Molecular pharmacology* **67**, 1600-1611

Potschka, H., Fedrowitz, M., and Loscher, W. (2003) Brain access and anticonvulsant efficacy of carbamazepine, lamotrigine, and felbamate in ABCC2/MRP2-deficient TR- rats. *Epilepsia* **44**, 1479-1486

Potschka, H., and Loscher, W. (2001) In vivo evidence for P-glycoprotein-mediated transport of phenytoin at the blood-brain barrier of rats. *Epilepsia* **42**, 1231-1240

Pramod, A. B.*, et al.* (2013) SLC6 transporters: structure, function, regulation, disease association and therapeutics. *Molecular aspects of medicine* **34**, 197-219

Pratt, S.*, et al.* (2005) The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. *Molecular cancer therapeutics* **4**, 855-863

Proks, P.*, et al.* (2002) Sulfonylurea stimulation of insulin secretion. *Diabetes* **51 Suppl 3**, S368-376

Pushpakom, S. P.*, et al.* (2011) Genetic variants of ABCC10, a novel tenofovir transporter, are associated with kidney tubular dysfunction. *The Journal of infectious diseases* **204**, 145-153

Reid, G.*, et al.* (2003) The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc.Natl.Acad.Sci.U.S.A* **100**, 9244-9249

Rigato, I.*, et al.* (2004) The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin. *The Biochemical journal* **383**, 335-341

Ringpfeil, F.*, et al.* (2000) Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. *Proc Natl Acad Sci U S A* **97**, 6001-6006

Riordan, J. R.*, et al.* (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-1073

Ritzel, M. W.*, et al.* (2001) Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *The Journal of biological chemistry* **276**, 2914-2927

Ritzel, M. W.*, et al.* (1997) Molecular cloning and functional expression of cDNAs encoding a human Na+-nucleoside cotransporter (hCNT1). *The American journal of physiology* **272**, C707-714

Ritzel, M. W.*, et al.* (1998) Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. *Molecular membrane biology* **15**, 203-211

Rius, M., Hummel-Eisenbeiss, J., and Keppler, D. (2008) ATP-dependent transport of leukotrienes B4 and C4 by the multidrug resistance protein ABCC4 (MRP4). *J.Pharmacol.Exp.Ther.* **324**, 86-94

Rius, M.*, et al.* (2003) Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. *Hepatology (Baltimore, Md.)* **38**, 374-384

Rizwan, A. N., and Burckhardt, G. (2007) Organic anion transporters of the SLC22 family: biopharmaceutical, physiological, and pathological roles. *Pharmaceutical research* **24**, 450-470

Rizzi, M.*, et al.* (2002) Limbic seizures induce P-glycoprotein in rodent brain: functional implications for pharmacoresistance. *J.Neurosci.* **22**, 5833-5839

Rommens, J. M.*, et al.* (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245**, 1059-1065

Roth, M., Obaidat, A., and Hagenbuch, B. (2011) OATPs, OATs and OCTs: The organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br.J.Pharmacol.*

Rowland, A.*, et al.* (2006) In vitro characterization of lamotrigine N2-glucuronidation and the lamotrigine-valproic acid interaction. *Drug metabolism and disposition: the biological fate of chemicals* **34**, 1055-1062

Sandtner, W.*, et al.* (2011) Ouabain binding site in a functioning Na+/K+ ATPase. *The Journal of biological chemistry* **286**, 38177-38183

Sandusky, G. E.*, et al.* (2002) Expression of multidrug resistance-associated protein 2 (MRP2) in normal human tissues and carcinomas using tissue microarrays. *Histopathology* **41**, 65-74

Satlin, L. M., Amin, V., and Wolkoff, A. W. (1997) Organic anion transporting polypeptide mediates organic anion/HCO3- exchange. *The Journal of biological chemistry* **272**, 26340-26345

Schaub, T. P.*, et al.* (1999) Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. *Journal of the American Society of Nephrology : JASN* **10**, 1159-1169

Scheffer, G. L.*, et al.* (2002a) MRP6 (ABCC6) detection in normal human tissues and tumors. *Laboratory investigation; a journal of technical methods and pathology* **82**, 515-518

Scheffer, G. L.*, et al.* (2002b) Tissue distribution and induction of human multidrug resistant protein 3. *Laboratory investigation; a journal of technical methods and pathology* **82**, 193-201

Schinkel, A. H.*, et al.* (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J.Clin.Invest* **97**, 2517-2524

Seelig, A. (1998) A general pattern for substrate recognition by P-glycoprotein. *Eur.J.Biochem.* **251**, 252-261

Shank, R. P.*, et al.* (1994) Topiramate: preclinical evaluation of structurally novel anticonvulsant. *Epilepsia* **35**, 450-460

Shi, Y. (2013) Common folds and transport mechanisms of secondary active transporters. *Annual review of biophysics* **42**, 51-72

Shin, H. J.*, et al.* (2007) Novel liver-specific organic anion transporter OAT7 that operates the exchange of sulfate conjugates for short chain fatty acid butyrate. *Hepatology (Baltimore, Md.)* **45**, 1046-1055

Siddiqui, A.*, et al.* (2003) Association of multidrug resistance in epilepsy with a polymorphism in the drug-transporter gene ABCB1. *N.Engl.J.Med.* **348**, 1442-1448

Sills, G. J.*, et al.* (2002) P-glycoprotein-mediated efflux of antiepileptic drugs: preliminary studies in mdr1a knockout mice. *Epilepsy Behav.* **3**, 427-432

Sills, G. J.*, et al.* (2005) Lack of association between the C3435T polymorphism in the human multidrug resistance (MDR1) gene and response to antiepileptic drug treatment. *Epilepsia* **46**, 643-647

Singh, S. K.*, et al.* (2008) A competitive inhibitor traps LeuT in an open-to-out conformation. *Science* **322**, 1655-1661

Sinz, M. W., and Remmel, R. P. (1991) Isolation and characterization of a novel quaternary ammonium-linked glucuronide of lamotrigine. *Drug metabolism and disposition: the biological fate of chemicals* **19**, 149-153

Sisodiya, S. M.*, et al.* (2002) Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. *Brain* **125**, 22-31

Smith, K. M.*, et al.* (2004) Electrophysiological characterization of a recombinant human Na+-coupled nucleoside transporter (hCNT1) produced in Xenopus oocytes. *The Journal of physiology* **558**, 807-823

Smith, K. M.*, et al.* (2007) Cation coupling properties of human concentrative nucleoside transporters hCNT1, hCNT2 and hCNT3. *Molecular membrane biology* **24**, 53-64

Smith, K. M.*, et al.* (2005) The broadly selective human Na+/nucleoside cotransporter (hCNT3) exhibits novel cation-coupled nucleoside transport characteristics. *The Journal of biological chemistry* **280**, 25436-25449

Spina, E., Pisani, F., and Perucca, E. (1996) Clinically significant pharmacokinetic drug interactions with carbamazepine. An update. *Clinical pharmacokinetics* **31**, 198-214

Struk, B.*, et al.* (2000) Mutations of the gene encoding the transmembrane transporter protein ABC-C6 cause pseudoxanthoma elasticum. *Journal of molecular medicine (Berlin, Germany)* **78**, 282-286

Sugano, K.*, et al.* (2010) Coexistence of passive and carrier-mediated processes in drug transport. *Nature reviews. Drug discovery* **9**, 597-614

Summerfield, S. G.*, et al.* (2007) Central nervous system drug disposition: the relationship between in situ brain permeability and brain free fraction. *J.Pharmacol.Exp.Ther.* **322**, 205-213

Takeda, M.*, et al.* (2002a) Characterization of methotrexate transport and its drug interactions with human organic anion transporters. *J Pharmacol Exp Ther* **302**, 666-671

Takeda, M.*, et al.* (2002b) Human organic anion transporters and human organic cation transporters mediate renal antiviral transport. *J Pharmacol Exp Ther* **300**, 918-924

Tamai, I.*, et al.* (1998) Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *The Journal of biological chemistry* **273**, 20378-20382

Tamai, I.*, et al.* (1997) Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. *FEBS letters* **419**, 107-111

Tammur, J.*, et al.* (2001) Two new genes from the human ATP-binding cassette transporter superfamily, ABCC11 and ABCC12, tandemly duplicated on chromosome 16q12. *Gene* **273**, 89-96

Taniguchi, K.*, et al.* (1996) A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. *Cancer Res* **56**, 4124-4129

Taubert, D.*, et al.* (2007) Identification of the endogenous key substrates of the human organic cation transporter OCT2 and their implication in function of dopaminergic neurons. *PloS one* **2**, e385

The Epilepsy Foundation of America (2013) Tonic-Clonic Seizures http://www.epilepsyfoundation.org/aboutepilepsy/seizures/genconvulsive/tonicseizures.cfm

Thorn, C. F.*, et al.* (2012) PharmGKB summary: phenytoin pathway. *Pharmacogenet Genomics* **22**, 466-470

Tishler, D. M.*, et al.* (1995) MDR1 gene expression in brain of patients with medically intractable epilepsy. *Epilepsia* **36**, 1-6

Tombline, G.*, et al.* (2005) Involvement of the "occluded nucleotide conformation" of P-glycoprotein in the catalytic pathway. *Biochemistry* **44**, 12879-12886

Tzvetkov, M. V.*, et al.* (2009) The effects of genetic polymorphisms in the organic cation transporters OCT1, OCT2, and OCT3 on the renal clearance of metformin. *Clinical pharmacology and therapeutics* **86**, 299-306

UCB Inc. (2013) Keppra Prescribing Information

Uchida, Y.*, et al.* (2011) Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *J.Neurochem.* **117**, 333-345

United States Food and Drug Administration (2012) Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations.

Urban, T. J.*, et al.* (2008) Effects of genetic variation in the novel organic cation transporter, OCTN1, on the renal clearance of gabapentin. *Clinical pharmacology and therapeutics* **83**, 416-421

Validus Pharmaceuticals LLC (2009) Equetro Prescribing Information

van Aubel, R. A.*, et al.* (2002) The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *Journal of the American Society of Nephrology : JASN* **13**, 595-603

Volk, C.*, et al.* (2009) Five amino acids in the innermost cavity of the substrate binding cleft of organic cation transporter 1 interact with extracellular and intracellular corticosterone. *Molecular pharmacology* **76**, 275-289

Walker, J. E.*, et al.* (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945-951

Wan, Q. F.*, et al.* (2010) SV2 acts via presynaptic calcium to regulate neurotransmitter release. *Neuron* **66**, 884-895

Wang, H.*, et al.* (2004) Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. *The Journal of biological chemistry* **279**, 29295-29301

Wang, P.*, et al.* (2008) Topological assessment of oatp1a1: a 12-transmembrane domain integral membrane protein with three N-linked carbohydrate chains. *American journal of physiology. Gastrointestinal and liver physiology* **294**, G1052-1059

Warren, M. S.*, et al.* (2009) Comparative gene expression profiles of ABC transporters in brain microvessel endothelial cells and brain in five species including human. *Pharmacol Res* **59**, 404-413

Watts, R. J., and Dennis, M. S. (2013) Bispecific antibodies for delivery into the brain. *Current opinion in chemical biology* **17**, 393-399

Weber, C., Fraemohs, L., and Dejana, E. (2007) The role of junctional adhesion molecules in vascular inflammation. *Nature reviews. Immunology* **7**, 467-477

Westlake, C. J., Cole, S. P., and Deeley, R. G. (2005) Role of the NH2-terminal membrane spanning domain of multidrug resistance protein 1/ABCC1 in protein processing and trafficking. *Molecular biology of the cell* **16**, 2483-2492

Wiebe, S.*, et al.* (2001) A randomized, controlled trial of surgery for temporal-lobe epilepsy. *The New England journal of medicine* **345**, 311-318

Wielinga, P.*, et al.* (2005) The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. *Cancer Res* **65**, 4425-4430

Wielinga, P. R.*, et al.* (2003) Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. *The Journal of biological chemistry* **278**, 17664-17671

Windass, A. S.*, et al.* (2007) The contribution of organic anion transporters OAT1 and OAT3 to the renal uptake of rosuvastatin. *J Pharmacol Exp Ther* **322**, 1221-1227

Woodward, O. M.*, et al.* (2009) Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proc Natl Acad Sci U S A* **106**, 10338-10342

World Health Organisation (2009) Epilepsy fact sheet no. 999 http://www.who.int/mediacentre/factsheets/fs999/en/index.html

Wu, Y.*, et al.* (2007) Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity. *The Journal of allergy and clinical immunology* **119**, 973-981

Xu, J.*, et al.* (2004) Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. *The Journal of biological chemistry* **279**, 19781-19789

Yabuuchi, H.*, et al.* (2001) Multiple splicing variants of two new human ATP-binding cassette transporters, ABCC11 and ABCC12. *Biochemical and biophysical research communications* **288**, 933-939

Yabuuchi, H.*, et al.* (1999) Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations. *J Pharmacol Exp Ther* **289**, 768-773

Yamada, A.*, et al.* (2007) Multiple human isoforms of drug transporters contribute to the hepatic and renal transport of olmesartan, a selective antagonist of the angiotensin II AT1-receptor. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 2166-2176

Yamamoto, T.*, et al.* (2007) Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters. *European journal of pharmacology* **557**, 1-8

Yamashita, A.*, et al.* (2005) Crystal structure of a bacterial homologue of Na+/Cl--dependent neurotransmitter transporters. *Nature* **437**, 215-223

Yang, Y., Mo, W., and Zhang, J. T. (2010) Role of transmembrane segment 5 and extracellular loop 3 in the homodimerization of human ABCC1. *Biochemistry* **49**, 10854-10861

Yip, V. L.*, et al.* (2012) HLA genotype and carbamazepine-induced cutaneous adverse drug reactions: a systematic review. *Clinical pharmacology and therapeutics* **92**, 757-765

Yoshiura, K.*, et al.* (2006) A SNP in the ABCC11 gene is the determinant of human earwax type. *Nature genetics* **38**, 324-330

Young, J. D.*, et al.* (2013) The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29. *Molecular aspects of medicine* **34**, 529-547

Zeng, H.*, et al.* (2000) Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Res* **60**, 4779-4784

Zhang, C.*, et al.* (2010) In vitro concentration dependent transport of phenytoin and phenobarbital, but not ethosuximide, by human P-glycoprotein. *Life Sci.* **86**, 899-905

Zhang, C.*, et al.* (2011) In vitro transport profile of carbamazepine, oxcarbazepine, eslicarbazepine acetate, and their active metabolites by human P-glycoprotein. *Epilepsia*

Zhang, L.*, et al.* (1997) Cloning and functional expression of a human liver organic cation transporter. *Molecular pharmacology* **51**, 913-921

Chapter 2

A comprehensive functional and clinical analysis of ABCC2 and its impact on treatment response to carbamazepine

Contents

2.1. Introduction

Note: Substantial parts of this research chapter have been published in the *The Pharmacogenomics Journal* (Radisch *et al.* 2014).

At the BBB, overexpression of ABCC2 (MRP2) has been suggested to be involved in CBZ treatment failure by lowering the drug concentration in the brain, particularly at the seizure focus (Aronica et al. 2004, Dombrowski et al. 2001, Kubota et al. 2006). So far, however, direct evidence for CBZ being a substrate for ABCC2 is lacking (Luna-Tortos *et al.* 2010).

A number of recently published clinical studies have suggested an association between CBZ treatment response and *ABCC2* genetic variants. In a study of Caucasian patients, a higher probability for CBZ treatment response was observed for the c.1249G>A variant (Ufer *et al.* 2011). The c.1249G>A variant was also reported to be related to CBZ adverse neurological drug reactions in a Korean case-control study (Kim *et al.* 2010). A recent study of Han Chinese epilepsy patients reported an association between treatment resistance and the c.1249G>A variant (Ma *et al.* 2014). However, these results were only significant in a pooled analysis of patients receiving multidrug therapy while a subgroup analysis of patients treated with CBZ/oxcarbazepine monotherapy remained non-significant (Ma *et al.* 2014). In an Indian population, seizure control in women treated with CBZ was associated with the variants -1549G>A and -1019A>G in the *ABCC2* promoter region (Grover *et al.* 2012). Another variant within the *ABCC2* promoter region, -24C>T, and the synonymous variant c.3972C>T have been reported to be associated with AED treatment resistance in a study with Chinese epilepsy patients (Qu *et al.* 2012). These results, however, have not been stratified according to the drug treatment used as responses to different AEDs were pooled. Similarly, the -24C>T variant has been associated with AED treatment failure in a study conducted with Caucasian epilepsy patients mainly treated with valproate (Ufer *et al.* 2009).

A number of clinical studies, including a recently published meta-analysis, have also failed to detect an association between the most commonly investigated

ABCC2 single nucleotide polymorphisms (SNPs) -24C>T, c.1249G>A, c.3972C>T and AED treatment response (Grover *et al.* 2013, Hilger *et al.* 2012, Kim *et al.* 2009, Kwan *et al.* 2011, Seo *et al.* 2008, Sporis *et al.* 2013).

A model of the ABCC2 membrane topology highlighting the two coding-region polymorphisms c.1249G>A (valine substituted by isoleucine at position 417) and c.3972C>T (synonymous variant with isoleucine at position 1324) is illustrated in Figure 2.1.

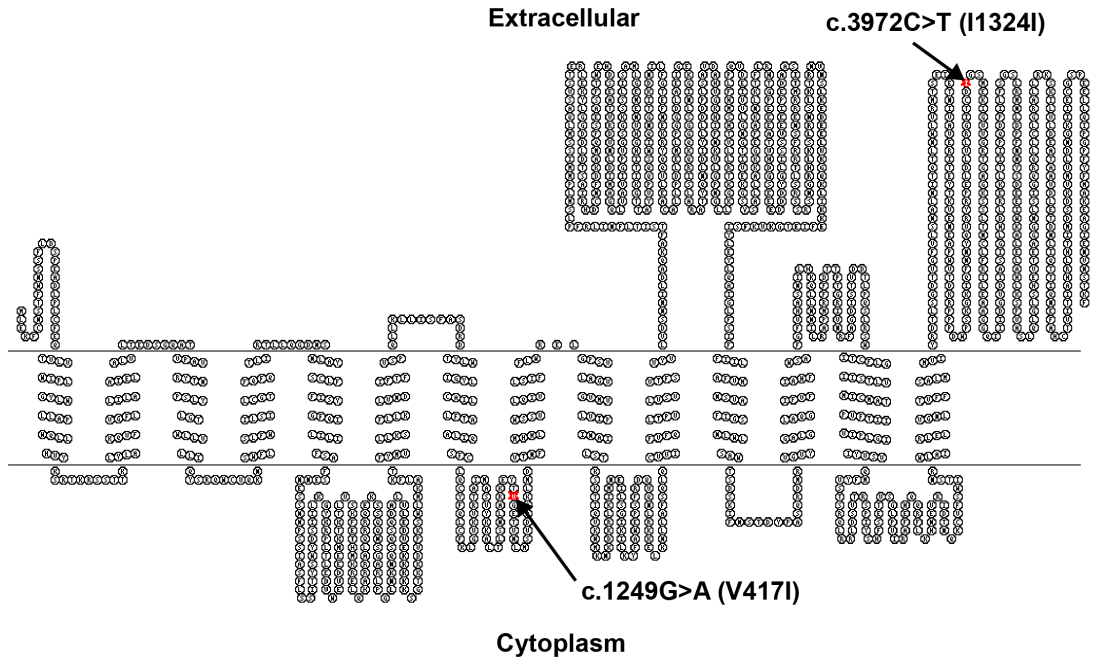


Figure 2.1: Predicted membrane topology of ABCC2

Membrane topology of ABCC2 as predicted by TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0) and plotted with TOPO2 (http://www.sacs.ucsf.edu/TOPO2). The corresponding amino acids from the coding genetic polymorphisms c.1249G>A (valine substituted by isoleucine at position 417) and c.3972C>T (synonymous polymorphism with isoleucine at position 1324) are highlighted in red

Due to the inconclusive and largely conflicting studies regarding the role of ABCC2 in CBZ treatment response, a comprehensive two-step analysis was undertaken. First, sets of *in vitro* assays were carried out to investigate whether CBZ was a substrate of ABCC2. Second, clinical and genetic data from the SANAD

(Standard and New Antiepileptic Drugs) study (Marson *et al.* 2007) were analysed focusing on three *ABCC2* gene polymorphisms, -24C>T, c.1249G>A, and c.3972C>T, and CBZ treatment response.

2.2. Materials and methods

2.2.1. Materials

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany. Dulbecco's modified eagle medium (DMEM), phosphate buffered saline without calcium/magnesium (PBS), and 0.05 % trypsin with ethylenediaminetetraacetic acid (EDTA) were obtained from Lonza GmbH, Cologne, Germany. Hanks' balanced salt solution (HBSS), HEPES (1 M) and fetal bovine serum (FBS) gold came from PAA Laboratories GmbH, Pasching, Austria. 0.25 % trypsin EDTA, MEM non-essential amino acids (MEM NEAA), penicillin (10,000 U)-streptomycin (10,000 µg/ml), L-glutamine (200 mM), and CellTracker™ Green 5-chloromethylfluorescein diacetate (CMFDA) were obtained from Life Technologies GmbH, Darmstadt, Germany. G418-BC (30,000 U/ml) was purchased from Biochrom AG, Berlin, Germany, Zeocin™ (100 mg/ml) from Cayla-InvivoGen Europe, Toulouse, France, and Aquasafe 300 Plus scintillation cocktail from Zinsser Analytic, Frankfurt, Germany. Tritium-labelled CBZ ([^{3}H]-CBZ, 1 mCi/ml, specific activity 10.0 Ci/mmol), tritium-labelled LTG ([^{3}H]-LTG, 1 mCi/ml, specific activity 5.0 Ci/mmol), tritium-labelled TPM ([^{3}H]-TPM, 1 mCi/ml, specific activity 8.0 Ci/mmol), and tritium-labelled LEV ([^{3}H]-LEV, 1 mCi/ml, specific activity 5.0 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc., St. Louis, MO, USA. For Western Blot, mouse anti-ABCC2 monoclonal antibody M_2III-6 and anti-mouse IgG (whole molecule)–peroxidase antibody produced in rabbit were purchased from Abcam plc, Cambridge, UK, and Sigma-Aldrich, Taufkirchen, Germany, respectively. Super Signal West Dura Extended Duration Substrate came from Thermo Fisher Scientific, Rockford, IL, USA.

2.2.2. Cell lines and culture conditions

2.2.2.1. Rht14-10 / Rht14-10 ABCC2(WT)-EGFP cell lines

Rht14-10 cells were derived from the human fibrosarcoma cell line HT1080 utilising a "screen and insert" strategy (Brough *et al.* 2007). The cells were stably transfected with a single insertion of the tetracycline-regulated *d2EGFP* reporter and the tetracycline transactivator protein. Cre-mediated recombination allowed site-specific insertion of a vector coding for ABCC2 (MRP2) tagged to enhanced green fluorescent protein (*ABCC2-EGFP.pEGFP-N1*) (Arlanov *et al.* 2012). Cells were cultured in DMEM supplemented with 10 % FBS (v/v), 0.4 % penicillin-streptomycin (v/v), 2 mM L-glutamine, 4 % MEM NEAA (v/v), and 0.2 % Zeocin (v/v). Rht14-10 ABCC2(WT)-EGFP cells were further supplemented with 0.8 % G418-BC (v/v). Both cell lines were cultured at 37 °C and 5 % CO_2.

2.2.2.2. MDCKII / MDCKII ABCC2 cell lines

Madin Darby canine kidney cells II (MDCKII) were used as parental cell line or stably transfected with *ABCC2* by means of retroviral transduction (kind gift from Prof. Dr. P. Borst, Netherland Cancer Institute, Amsterdam, NL) (Evers *et al.* 1998). Cells were cultured in DMEM supplemented with 10 % FBS (v/v) and 1 % penicillin-streptomycin (v/v) at 37 °C and 5 % CO_2.

2.2.3. Efflux assays

Cellular efflux assays were carried out as described previously with minor modifications (Arlanov *et al.* 2012). 200,000 cells/well were seeded onto Nunc™ 6-well plates (Fisher Scientific GmbH, Schwerte, Germany) and cultured for 3 days to obtain 80-90 % confluence. Cells were pre-incubated for 1 hour at 4 °C with either 5 μM CMFDA or 5 μM [^{3}H]-CBZ (2.4 μCi/ml) in transport buffer (HBSS supplemented with 10 mM HEPES, adjusted to pH 7.4). CMFDA, a non-fluorescent and membrane permeable chemical, was utilised as the positive control for ABCC2-mediated transport. Upon uptake into cells, CMFDA is hydrolysed by intra-cellular esterases and glutathione S-transferase into the non-membrane permeable, fluorescent

ABCC2 (MRP2) substrate glutathione-methylfluorescein (GS-MF) (Pratt *et al.* 2006, Roelofsen *et al.* 1997).

Radioassays with [^{3}H]-CBZ (2.4 μCi/ml) were supplemented with non-radiolabelled drug to a final concentration of 5 μM. For analysis, 100 μl samples were taken immediately (referred to as time point 0) and subsequently at different time points. Fluorescence emitted from the ABCC2 substrate GS-MF was determined with an excitation and emission wavelength of 485 nm and 535 nm, respectively (1420 Victor2 multilabel plate counter, Wallac, Turku, Finland). Radioactivity was determined by adding 5 ml scintillation cocktail to each sample with scintillation counting performed to detect radionuclide disintegrations per minute (HIDEX 300 SL Liquid Scintillation Counter, FCI Frenzel Consulting & Instruments, Straubenhardt, Germany).

2.2.4. Vesicle preparation

Plasma membrane vesicles were prepared from Rht14-10 and Rht14-10 ABCC2(WT)-EGFP cells based on a previously published protocol (Keppler *et al.* 1998) and is outlined below. Cells from each cell line were seeded on cell culture dishes of size 150 x 20 mm (Sarstedt AG & Co., Nümbrecht, Germany) with 15 ml medium per plate and incubated for three days to reach 80-90 % confluence.

2.2.4.1. Washing of cells

On the day of vesicle preparation, cells were scraped off the cell culture dishes to give about 400 ml cell suspension per cell line. Cell suspensions were centrifuged at 3,000 rpm for 10 minutes at 4 °C (Sorvall superspeed RC2-B centrifuge with GSA rotor, Fisher Scientific GmbH, Schwerte, Germany) and resuspended in 50 ml ice-cold PBS. The suspension was centrifuged at 1,500 rpm for 10 minutes at 4 °C (Universal centrifuge 320 R with swing-out rotor 1324, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany), again resuspended in 50 ml PBS and centrifuged as before.

2.2.4.2. Hypotonic cell lysis

The washed cell pellets were lysed in 95 ml hypotonic buffer (0.5 mM sodium phosphate, pH 7.4) containing the protease inhibitors phenylmethylsulfonyl fluoride (100 μM), aprotinin (0.3 μM), and leupeptin (1 μM). Cells were lysed for 1.5 hours under stirring (250 rpm) at 4 °C and the lysate subsequently centrifuged at 35,000 rpm for 45 minutes at 4 °C (Optima™ L-100 XP ultracentrifuge with type 70 Ti rotor, Beckman Coulter GmbH, Krefeld, Germany).

2.2.4.3. Plasma membrane isolation

Each cell pellet was resuspended in 10 ml hypotonic buffer and homogenised with 30 strokes at 500 rpm and 4 °C using an electric Potter-Elvehjem homogeniser model S (Sartorius AG, Göttingen, Germany). The homogenised cell lysates were centrifuged at 10,000 rpm for 10 minutes at 4 °C (Sorvall superspeed RC2-B centrifuge with SS34 rotor, Fisher Scientific GmbH, Schwerte, Germany) and the supernatant stored on ice. The pellets were resuspended in 7.5 ml incubation buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) with protease inhibitors and again homogenised (20 strokes) and centrifuged as before. Supernatants from both postnuclear homogenisation steps were finally combined and centrifuged at 35,000 rpm for 45 minutes at 4 °C (ultracentrifuge as in 2.2.4.2).

2.2.4.4. Plasma membrane purification and vesicle preparation

The resulting cell pellets from the plasma membrane isolation step were resuspended in 15 ml incubation buffer with protease inhibitors followed by a manual homogenisation step with 50 strokes on ice using a Dounce type Potter-Elvehjem hand homogeniser (Sartorius AG, Göttingen, Germany). The homogenate was carefully transferred on top of a 38 % (w/v) sucrose solution (in 5 mM HEPES-KOH, pH 7.4) and centrifuged at 40,000 rpm for 1.5 hours at 4 °C (Optima™ L-100 XP ultracentrifuge with SW40 swing rotor, Beckman Coulter GmbH, Krefeld, Germany). The interphases (plasma membranes) and the pellets (endoplasmic reticulum) were removed separately and diluted with ice-cold incubation buffer without protease inhibitors to give a final volume of 10 ml each. Another manual

homogenisation step was carried out (20 strokes) and the homogenates subsequently centrifuged at 35,000 rpm for 50 minutes at 4 °C (Optima^{TM} L-100 XP ultracentrifuge with type 70 Ti rotor, Beckman Coulter GmbH, Krefeld, Germany). Each cell pellet was resuspended in 200 µl ice-cold incubation buffer without protease inhibitors. To generate vesicles, the suspensions were passed through a 27-gauge needle (20 times) (B. Braun Melsungen AG, Melsungen, Germany) and 20 µl aliquots were frozen in liquid nitrogen until needed. The protein concentration was determined according to the Smith method (Smith *et al.* 1985) and a confirmatory immunoblot carried out as described by Arlanov *et al.* (2012).

2.2.5. Vesicle uptake assays

Vesicle uptake assays were performed based on the rapid filtration method (Leier *et al.* 1994). Frozen vesicles were thawed at 37 °C and immediately used for uptake assays. Each reaction consisted of vesicles with 30 µg protein, 4 mM ATP or 4 mM AMP, 20 mM $MgCl_2$, 50 µM 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate mixed isomers (CDCF) as positive control or 5 µM [^{3}H]-CBZ, 5 µM [^{3}H]-LTG, 5 µM [^{3}H]-TPM, 5 µM [^{3}H]-LEV (0.6 µCi/ml) in transport buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4). Radioassays were supplemented with non-radiolabelled drug to give the final concentration of 5 µM.

Reactions were incubated at 37 °C in a water bath for 20 minutes and subsequently stopped by adding 1 ml ice-cold transport buffer. Reactions were then transferred and passed under vacuum (0.2 bar) through pre-wetted Millipore Durapore® membrane filters (pore size 0.22 µm) on a Millipore Sampling Manifold (Merck KGaA, Darmstadt, Germany). Filters were washed three times with ice-cold transport buffer and membrane vesicles finally lysed with 1 ml sodium dodecyl sulfate (SDS)-HEPES buffer (1 % SDS, 7.5 mM HEPES, pH 7.4) for 30 minutes at room temperature. 200 µl aliquots were taken and fluorescence determined (see 2.2.3). Filters with vesicles containing radiolabelled AEDs were transferred into a scintillation tube and incubated with 5 ml scintillation cocktail for 30 minutes before radioactivity was determined (see 2.2.3).

2.2.6. SANAD study details

The SANAD study was an unblinded randomised controlled clinical trial conducted in the UK between 1999 and 2005 (International Standard Randomised Controlled Trial number ISRCTN38354748). 1721 patients with partial epilepsy were randomised in arm A to CBZ, gabapentin, LTG, oxcarbazepine, or TPM, aiming to compare the long-term efficacy of the drugs. Details of the study have been described elsewhere (Marson *et al.* 2007). All patients gave their written informed consent to be included into the SANAD study and the study received all required approvals from ethics and research committees. Patients were followed for a minimum of 12 months and seizure outcomes included time to first seizure and time to 12-month seizure remission.

2.2.7. Genotyping

Genotyping was performed on Illumina 660 and received all required ethical approvals. All patients gave their written informed consent to be included into the study (Speed *et al.* 2013).

2.2.8. Patient selection

Patient selection from the SANAD study arm A was carried out in two steps. First, only patients with available genotyping data (678 patients) were selected. In a second step, patients from this group were eligible for inclusion if they had been treated with CBZ monotherapy for any time throughout the study. This included patients not necessarily initially randomised to CBZ. A transition period with overlapping treatments was considered acceptable when followed by CBZ monotherapy (tapering of previous medication). Three patients with an exception made from above criteria have been included. These patients were on CBZ monotherapy for 1737 days, 1967 days, and 1299 days, respectively. Within this period, records indicated additional TPM intake between two subsequent visits of 77 days, 103 days, and 273 days, respectively. The intake of CBZ remained

unchanged for the whole period. After the selection process, a total of 229 patients were eligible for statistical analysis.

2.2.9. Statistical analysis

Survival analysis was undertaken utilising the 'survival' package in R (version 3.0.0). Polymorphisms rs717620 (-24G>A), rs2273697 (c.1249G>A), and rs3740067 (c.3843+124C>G) were analysed for two outcomes, time to first seizure and time to 12-month remission. All three polymorphisms were found to be in Hardy-Weinberg equilibrium. Data from patients without the outcome event having occurred before a stop date were included as right-censored data. A stop date was defined as addition of another AED to CBZ monotherapy treatment, non-compliance, loss to follow-up, withdrawn consent, and end of study. The three polymorphisms were stratified according to genotype but minor allele homozygotes were pooled with heterozygotes because of their low frequency.

Kaplan-Meier survival probabilities were calculated and curves plotted for each condition (stepped curves). In addition, parametric regression models were fitted to the data. Parametric survival models describe the outcomes based on a known distribution and therefore are smooth curves rather than stepped curves (Kleinbaum *et al.* 2012). Curves derived from a parametric survival distribution approach zero for an infinite time while Kaplan-Meier curves may not, due to patients still at risk at the end of the study (Kleinbaum *et al.* 2012). Three commonly used distributions, exponential, Weibull, and log-logistic, were evaluated for their appropriateness of fit to the survival data. Holding of model assumptions was explored graphically by transforming each parametric survival function and plotting the transformed Kaplan-Meier estimates against time or natural logarithm (ln) of time (Kleinbaum *et al.* 2012). A linear relationship with parallel lines indicated holding of model assumptions. For the final model selection, Akaike´s information criterion was calculated as a measure of the goodness of fit. The lowest number indicated the best fit. A p-value was obtained for the regression coefficients with $p < 0.05$ considered statistically significant.

In vitro efflux assays were carried out three independent times in triplicate. Data are presented as means ± standard deviation (SD). An independent two-tailed t-test was applied for statistical analysis of each time point (ABCC2 over-expressing vs. control cell lines). *In vitro* vesicle uptake experiments were carried out once in triplicate. Data are presented as means ± SD. A one-way analysis of variance (ANOVA) was performed for statistical analysis followed by a Dunnett's post-hoc test for significant results with $p<0.05$. Significant results are indicated with * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$. Analysis was performed with SPSS® Statistics version 20 (IBM United Kingdom Ltd., Hampshire, UK).

2.3. Results

2.3.1. *In vitro* cell efflux assays

In initial experiments, time-course cell efflux assays were undertaken to study CBZ as a potential substrate for ABCC2 (MRP2). CMFDA served as a positive control pro-drug and confirmed ABCC2 activity in both transfected cell lines (Figure 2.2 A+B). No increased CBZ efflux was observed from *ABCC2*-transfected Rht14-10 and MDCKII cells as compared to controls (Figure 2.2 C+D).

As CBZ has previously been reported to inhibit ABCC2-mediated GS-MF efflux (Kim *et al.* 2010), the same assay was repeated in the presence of 100 µM CBZ or vehicle. No difference in GS-MF efflux was observed between CBZ or vehicle-only treated cells (Figure 2.3).

2.3.2. *In vitro* vesicle uptake assays

To further validate the results obtained from cell efflux assays, inside-out plasma membrane vesicles were prepared from Rht14-10 and Rht14-10 ABCC2(WT)-EGFP cells. An immunoblot confirmed the presence and absence of ABCC2-EGFP, respectively (Figure 2.4).

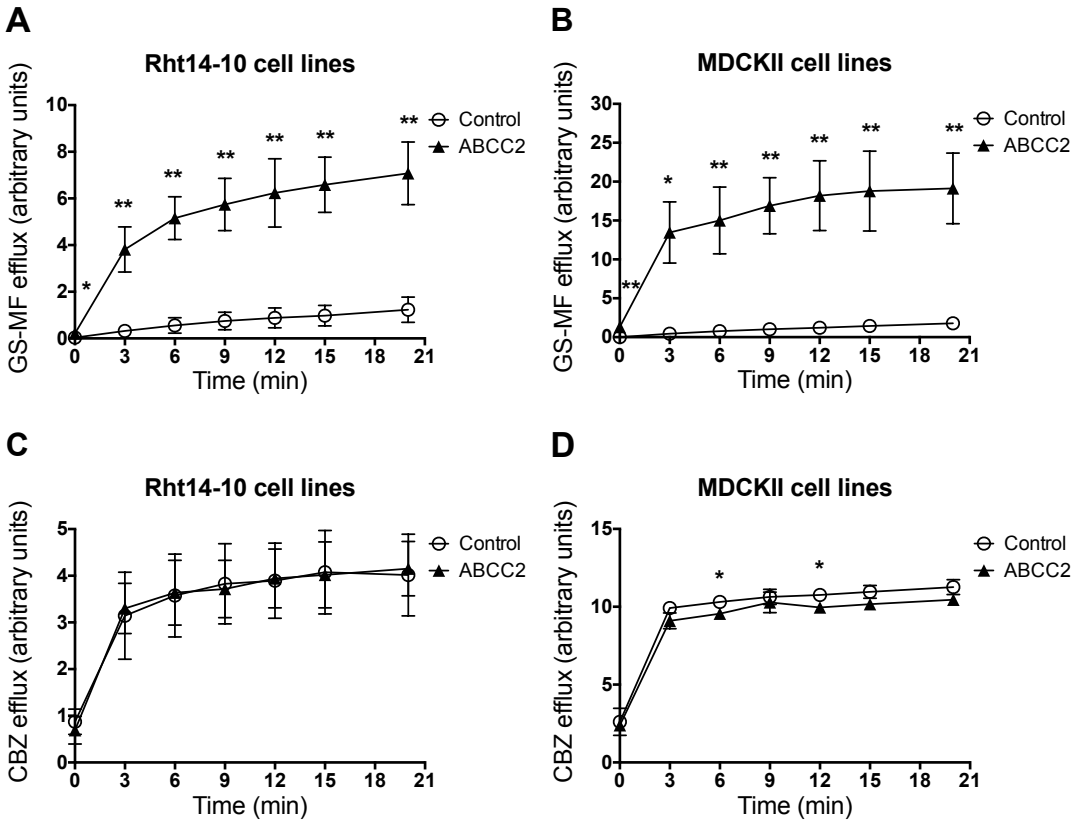


Figure 2.2: Cell efflux assays to investigate whether carbamazepine is an ABCC2 substrate

Time-course efflux assays from human fibrosarcoma (Rht14-10) and Madin Darby canine kidney II (MDCKII) cell lines. Efflux was compared between cells stably transfected with *ABCC2* and controls (Rht14-10 ABCC2(WT)-EGFP vs. Rht14-10 and MDCKII ABCC2 vs. MDCKII). A+B) Positive control substrate glutathione-methylfluorescein (GS-MF); C+D) Carbamazepine (CBZ). Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with * for $p<0.05$ and ** for $p<0.01$

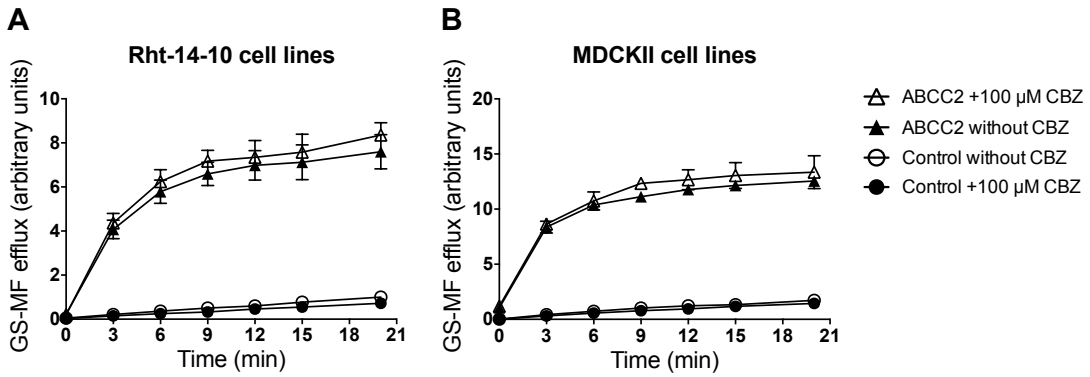


Figure 2.3: Cell efflux competition assays to investigate whether carbamazepine is an ABCC2 inhibitor

Time-course efflux assays from A) human fibrosarcoma (Rht14-10) and B) Madin Darby canine kidney II (MDCKII) cell lines. Efflux of glutathione-methylfluorescein (GS-MF) was compared between cells stably transfected with *ABCC2* and controls (Rht14-10 ABCC2(WT)-EGFP vs. Rht14-10 and MDCKII ABCC2 vs. MDCKII) in the presence or absence of 100 µM carbamazepine (CBZ). Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate)

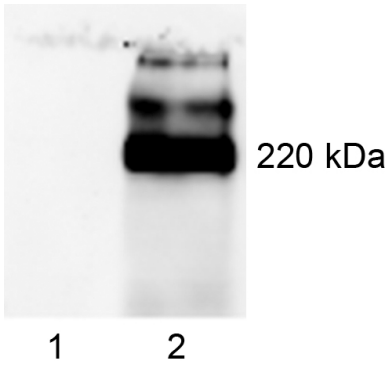


Figure 2.4: ABCC2 immunoblot of plasma membrane vesicle preparations

Immunoblot with monoclonal antibody against ABCC2 (MRP2) indicating expression or absence of ABCC2-EGFP. Lane 1: Rht14-10 plasma membrane vesicles; Lane 2: Rht14-10 ABCC2(WT)-EGFP plasma membrane vesicles

CDCF served as a positive control substrate and is closely related to the CMFDA hydrolysis product GS-MF (Pratt *et al.* 2006). Drug uptake was determined in the presence of either ATP or AMP. ABCC2 (MRP2) over-expressing vesicles exhibited a significant 94 % reduction of CDCF uptake when ATP was replaced by AMP (Figure 2.5 A). In addition, control vesicles accumulated 26 % less CDCF in the presence of ATP as compared to ABCC2 over-expressing vesicles. These results confirmed that vesicle preparations were functionally suitable to investigate CBZ as a potential ABCC2 substrate. In contrast to CDCF, CBZ uptake into control and ABCC2-EGFP over-expressing vesicles was not different both in the presence of AMP or ATP (Figure 2.5 B).

In addition to CBZ, the uptake of three other AEDs, namely LTG, TPM, and LEV, was analysed but also with negative outcome (Figure 2.5 C-E). Although the focus of this research chapter was the analysis of CBZ, the additional three AEDs were included because only limited transport studies on ABCC2 have been done so far (Baltes *et al.* 2007, Luna-Tortos *et al.* 2010, Luna-Tortos *et al.* 2009). In agreement with the negative results presented here, these studies also failed to find any ABCC2-mediated transport of LTG, TPM, and LEV and thus further experiments were not pursued.

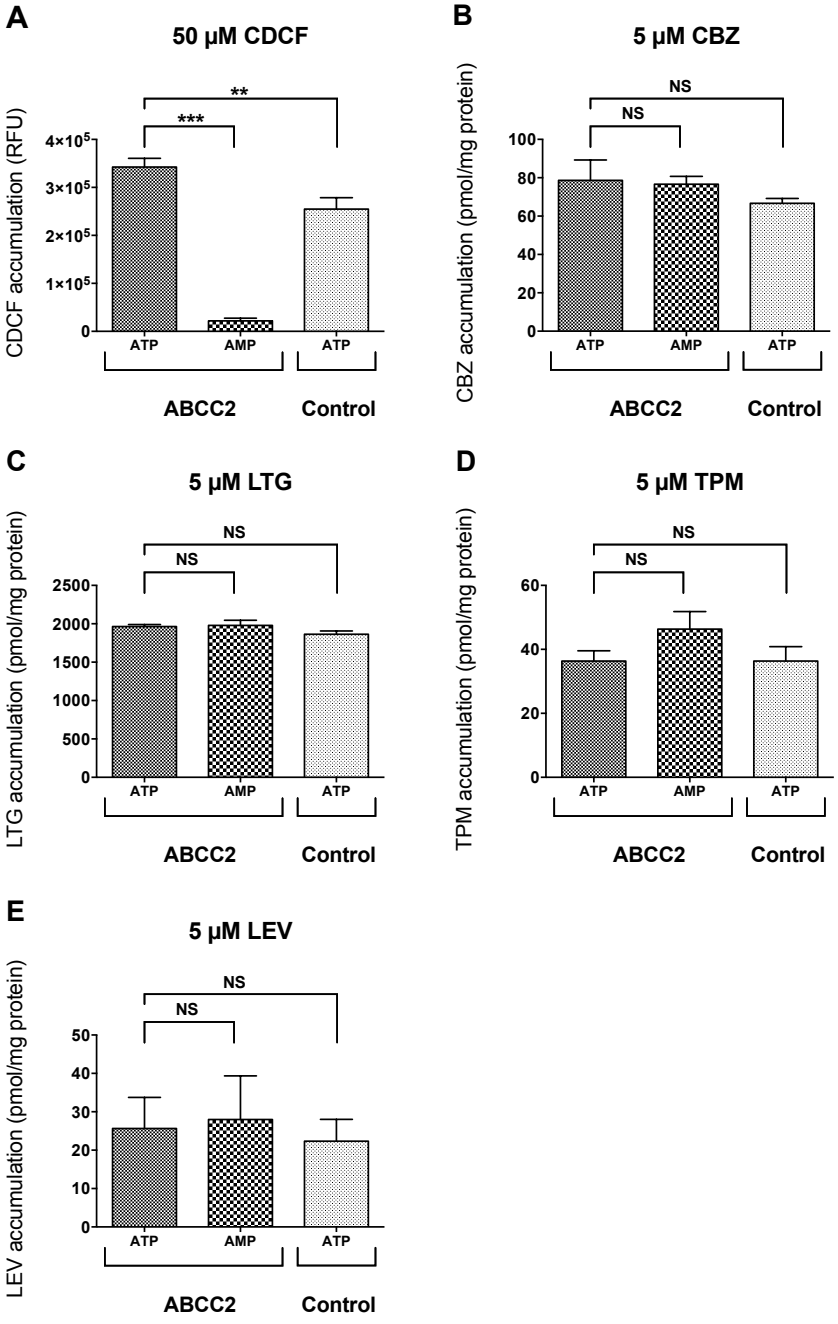


Figure 2.5: Vesicle uptake assays to investigate whether different antiepileptic drugs are substrates of ABCC2

Uptake assays into inside-out vesicles derived from human fibrosarcoma cell lines stably over-expressing ABCC2-EGFP (Rht14-10 ABCC2(WT)-EGFP) and controls (Rht14-10). Assays were carried out in the presence of either 4 mM ATP or 4 mM AMP and uptake determined after 20 minutes; A) Positive control substrate 50 µM 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate mixed isomers (CDCF); B) 5 µM carbamazepine (CBZ); C) 5 µM lamotrigine (LTG); D) 5 µM topiramate (TPM); E) 5 µM levetiracetam (LEV). Data are expressed as means ± standard deviation (n = 3). Significant results are indicated with ** for $p<0.01$ and *** for $p<0.001$. NS = Non-significant

2.3.3. Clinical analysis

The clinical impact of three *ABCC2* polymorphisms on CBZ treatment outcomes (time to first seizure and time to 12-month remission) was analysed using samples from the SANAD study. 229 patients on CBZ monotherapy were eligible for analysis of time to first seizure. Out of these, a subset of 134 patients received treatment for at least 365 days and was eligible for analysis of time to 12-month remission. The clinical and demographic data are summarised in Table 2.1.

Table 2.1: Baseline clinical and demographic characteristics for patients selected for time to first seizure (n=229) and time to 12-month remission outcome analysis (n=134)

		Time to first seizure (n=229)	**Time to 12-month remission (n=134)**
Sex			
	Female	113 (49 %)	72 (54 %)
Treatment history			
	Untreated	115 (50 %)	74 (55 %)
History			
	Learning disability	16 (7 %)	8 (6 %)
	Neurological deficit	17 (7 %)	8 (6 %)
Neurological disorder			
	Stroke/cerebrovascular	19 (8 %)	14 (10 %)
	Intracranial surgery	12 (5 %)	7 (5 %)
	Head injury	9 (4 %)	6 (4 %)
	Meningitis/encephalitis	5 (2 %)	2 (1 %)
	Other	18 (8 %)	12 (9 %)
Epilepsy syndrome			
	Partial	215 (94 %)	122 (91 %)
	Generalised	0 (0 %)	0 (0 %)
	Unclassified	14 (6 %)	12 (9 %)
Mean age in years at CBZ treatment start (SD)		41 (17)	42 (17)

Genetic polymorphisms of interest included rs717620 (-24C>T), rs2273697 (c.1249G>A), and rs3740066 (c.3972C>T). The Illumina chip covered the first two variants but not the latter one. The closely located SNP rs3740067 (c.3843+124C>G), however, was included on the chip and is in perfect linkage disequilibrium (LD) to rs3740066 (r^2=1, Figure 2.6). It was therefore eligible as a surrogate marker. Minor allele frequencies (MAFs) for all three polymorphisms are summarised in Table 2.2 and correspond well to the MAFs extracted from HapMap. All HapMap data used for analysis were from Utah residents with northern and western European ancestry (CEU) from phase III release 28.

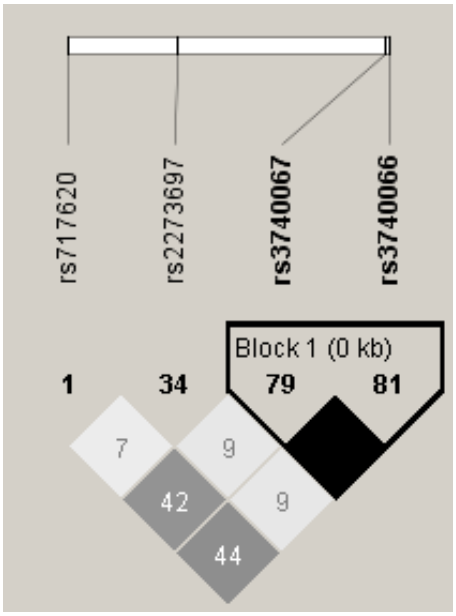


Figure 2.6: Linkage disequilibrium plot for selected *ABCC2* genetic polymorphisms

Linkage disequilibrium (LD) plot for the genetic polymorphisms rs717620 (-24C>T), rs2273697 (c.1249G>A), rs3740066 (c.3972C>T), and the surrogate marker rs3740067 (c.3843+124C>G). LD is given as r^2 with the black box indicating perfect correlation (r^2=1)

Table 2.2: Minor allele frequencies for selected polymorphisms in *ABCC2*

rs717620 (-24G>A)		**rs2273697 (c.1249G>A)**		**rs3740067 (c.3843+124C>G)**	
HapMap (CEU): 0.21		*HapMap (CEU): 0.24*		*HapMap (CEU): 0.35*	
Time to first seizure (n=229)	Time to 12-month remission (n=134)	Time to first seizure (n=229)	Time to 12-month remission (n=134)	Time to first seizure (n=228)	Time to 12-month remission (n=133)
0.20	0.18	0.18	0.19	0.38	0.35

SNPs rs717620 (-24C>T) and rs2273697 (c.1249G>A) were successfully genotyped in all 229 patients. rs3740067 (c.3843+124C>G) was successfully genotyped in 228 out of 229 patients. For survival analysis, each polymorphism was stratified against genotype and analysed for both outcomes, time to first seizure and time to 12-month remission. Kaplan-Meier curves were plotted from Kaplan-Meier estimates but frequently crossed each other indicating a violation of the proportional hazards assumption underlying Cox regression modelling and the log-rank test. To obtain a reliable p-value, additional parametric survival curves were fitted to the Kaplan-Meier estimates. Three commonly used distributions were assessed for their appropriateness to fit the Kaplan-Meier estimates, the exponential, Weibull, and log-logistic distribution. Holding of model assumptions was verified graphically by transforming each survival function into a linear function and plotting the transformed Kaplan-Meier estimates against time or natural logarithm (ln) of time (Table 2.3). Reasonable linear and parallel lines were obtained for the Weibull and log-logistic distributions for both outcomes analysed (Figure 2.7 and Figure 2.8).

Table 2.3: Parametric survival functions with linear transformations

Distribution	Survival function	Linear transformation of survival function
Exponential	$S(t) = \exp(-\lambda t)$	$-ln[S(t)] = \lambda t$
Weibull	$S(t) = \exp(-\lambda t^p)$	$ln[-\ln(S(t))] = \ln(\lambda) + p \cdot ln(t)$
Log-logistic	$S(t) = \frac{1}{1+\lambda t^p}$	$\ln\left(\frac{1}{S(t)} - 1\right) = p \cdot \ln(\lambda) + p \cdot \ln(t)$

For final model selection, Akaike´s information criterion was calculated as a measure of the goodness of fit. The log-logistic distribution was found to describe all analysed outcomes and genotypes best. With one exception, the p-values of the corresponding regression coefficients indicated no statistically significant differences between genotypes and outcomes (Figure 2.9). The time to first seizure analysis for SNP rs717620 (-24C>T) was statistically significant with p=0.034 but did not withstand Bonferroni correction for multiple testing ($p_c = 0.01$).

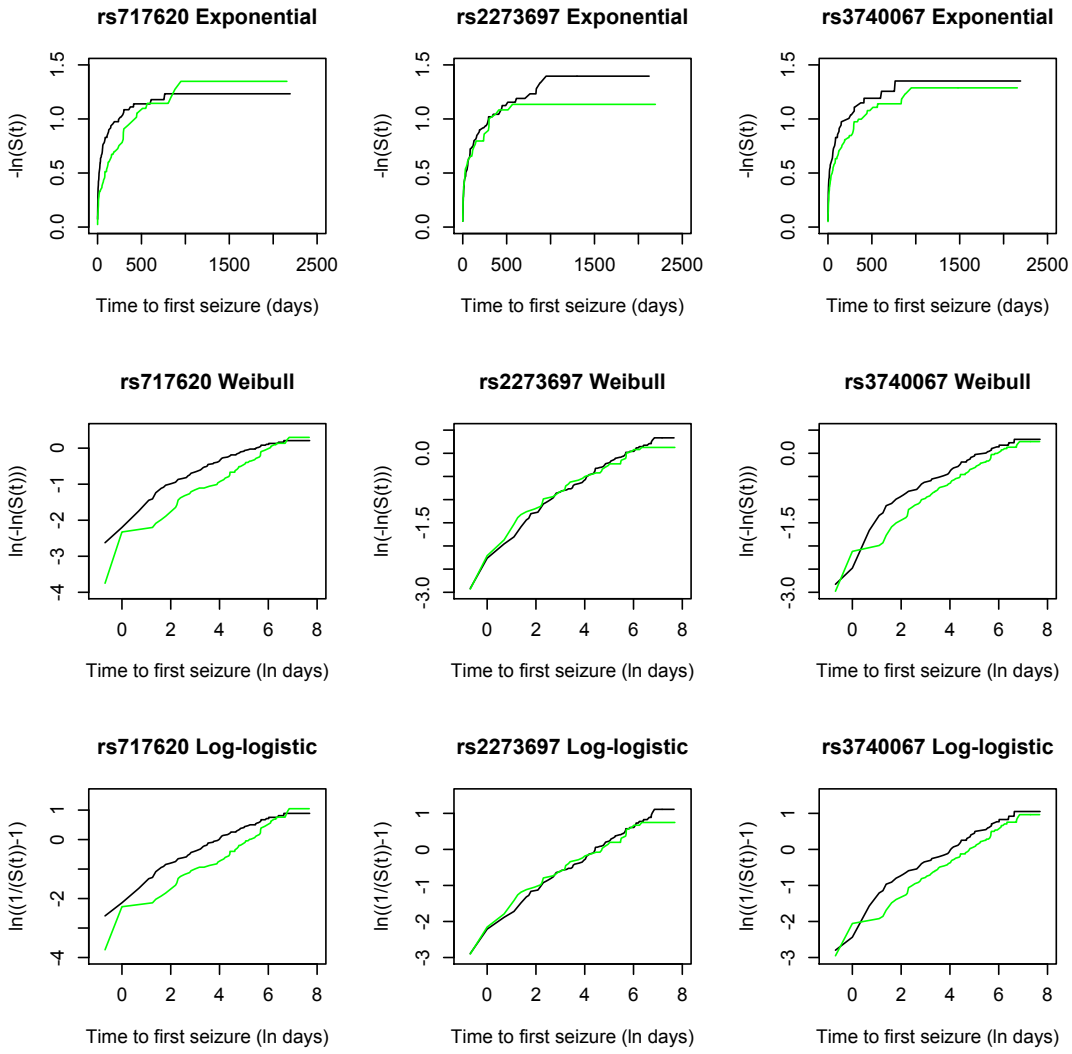


Figure 2.7: Graphical analysis of parametric model assumptions for time to first seizure outcomes

Transformed Kaplan-Meier estimates for genetic polymorphisms rs717620 (-24G>A), rs2273697 (c.1249G>A), and rs3740067 (c.3843+124C>G) stratified according to genotype are plotted against time or natural logarithm (ln) of time. Three commonly used distributions, exponential, Weibull, and log-logistic, were analysed for holding of model assumptions

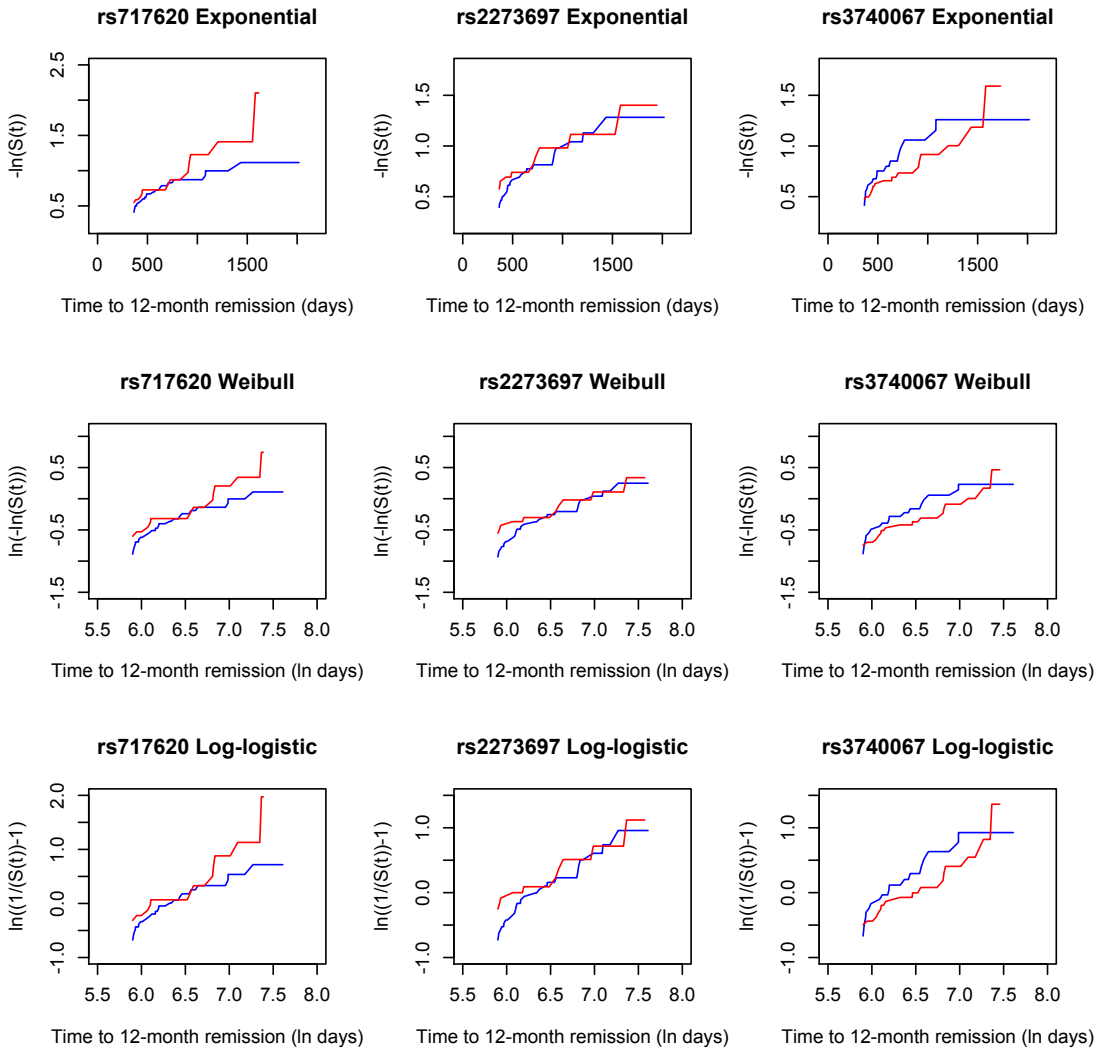
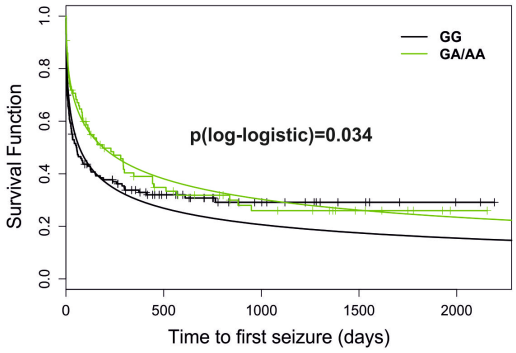


Figure 2.8: Graphical analysis of parametric model assumptions for time to 12-month remission outcomes

Transformed Kaplan-Meier estimates for genetic polymorphisms rs717620 (-24G>A), rs2273697 (c.1249G>A), and rs3740067 (c.3843+124C>G) stratified according to genotype are plotted against time or natural logarithm (ln) of time. Three commonly used distributions, exponential, Weibull, and log-logistic, were analysed for holding of model assumptions

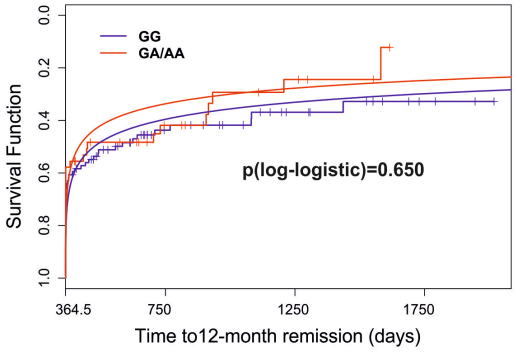
A



Numbers at risk

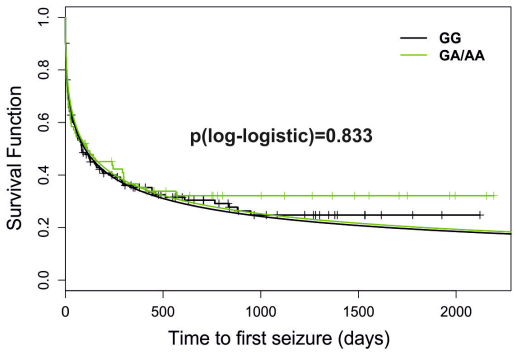
Day	0	100	250	500	1000	1500	2000
GG	143	60	48	30	14	6	2
GA/AA	86	49	35	24	13	7	1

B



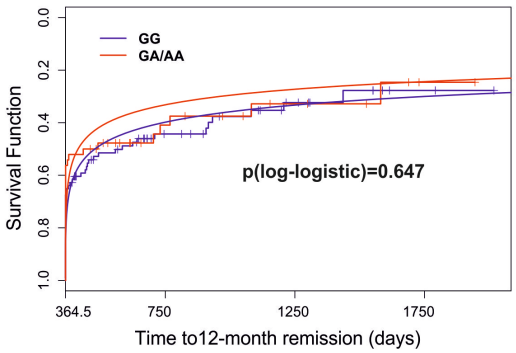
Numbers at risk

Day	364.5	500	750	1250	1750
GG	89	41	23	11	3
GA/AA	45	19	12	5	0



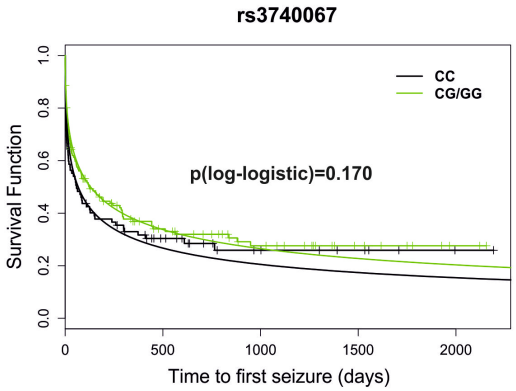
Numbers at risk

Day	0	100	250	500	1000	1500	2000
GG	152	70	53	33	15	6	1
GA/AA	77	39	30	21	12	7	2



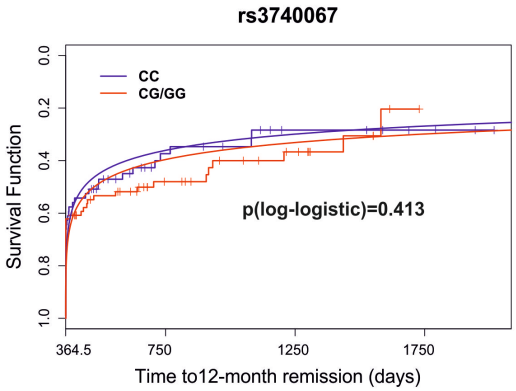
Numbers at risk

Day	364.5	500	750	1250	1750
GG	86	39	23	10	2
GA/AA	48	21	12	6	1



Numbers at risk

Day	0	100	250	500	1000	1500	2000
CC	87	38	31	19	8	5	1
CG/GG	141	70	51	34	18	8	2



Numbers at risk

Day	364.5	500	750	1250	1750
CC	59	25	14	6	3
CG/GG	74	35	21	10	0

Figure 2.9: Survival analysis for selected *ABCC2* genetic polymorphisms to investigate an impact on carbamazepine treatment response

Survival analysis for genetic polymorphisms rs717620 (-24G>A), rs2273697 (c.1249G>A), and rs3740067 (c.3843+124C>G) as a surrogate marker for rs3740066 (c.3972C>T) stratified according to genotype. Outcomes analysed were A) Time to first seizure and B) Time to 12-month remission. Step curves were plotted from Kaplan-Meier estimates with vertical tick-marks indicating right censoring. Smooth curves were derived from fitting a parametric log-logistic model to the data. The p-value obtained from the log-logistic regression coefficient is indicated. $p<0.05$ was considered statistically significant

2.4. Discussion

The study of ABCC2 (MRP2) as a potential mechanism for CBZ pharmacoresistance has become a highly active field of research but results from *in vitro* and clinical studies have largely been conflicting. The present analysis attempted to comprehensively investigate both aspects to gain more confidence in the potential importance of ABCC2 regarding CBZ treatment response in epilepsy.

Sets of highly sensitive *in vitro* assays were utilised to investigate the direct transport of tritium-labelled CBZ by ABCC2. CBZ was found to be negative in time-course cell efflux assays from two different *ABCC2* transfected cell lines (human Rht14-10 and canine MDCKII, Figure 2.2). In addition, 100 µM CBZ had no effect on ABCC2-mediated GS-MF efflux (Figure 2.3).

Efflux assays from cells can be limited by the initial uptake of the drug into the cells. To circumvent any potential uptake issues and metabolism with living cells, inside-out plasma membrane vesicles were prepared from Rht14-10 and Rht14-10 ABCC2(WT)-EGFP cell lines. The EGFP-tag has previously been shown not to affect ABCC2 function using two model substrates (Arlanov *et al.* 2012) and allows a visual control of protein expression throughout cell passaging. CDCF is a well-established control substrate for vesicular uptake assays with ABCC2 (Colombo *et al.* 2013, Heredi-Szabo *et al.* 2008, Kidron *et al.* 2012, Lechner *et al.* 2010) and mimics GS-MF, the hydrolysis and conjugation product of CMFDA used for cell efflux assays. Uptake of CDCF into ABCC2 over-expressing vesicles markedly dropped

when ATP was replaced by AMP (Figure 2.5 A). Also, control vesicles exhibited a reduced accumulation in the presence of ATP as compared to ABCC2 over-expressing vesicles. The remaining accumulation might be a result of compensatory effects by other, endogenous ABC transporters recognising that CDCF is a rather unspecific substrate (Lehmann *et al.* 2001, Pratt *et al.* 2006). The ABCC2 model substrates leukotriene C4 or estradiol-17β-D-glucuronide ($E_2$17βG) are not considered promising alternatives to CDCF, as both are also known substrates for other ABC transporters (see chapter 1 section 1.2.2). However, given that CDCF-uptake was strongly ATP-dependent but CBZ was not, CBZ does not seem to be a substrate for any of the expressed ABC transporters, including ABCC2 (Figure 2.5 B). Together, the presented negative cell-efflux and vesicle-uptake experiments do not suggest a role for ABCC2 in CBZ efflux.

The presented *in vitro* data confirm previously published results indicating no direct transport of CBZ by ABCC2 (Luna-Tortos *et al.* 2010) but conflict with the conclusions drawn by Kim *et al.* (2010) who performed indirect functional studies with human embryonic kidney cells (HEK293) over-expressing ABCC2 WT and the c.1249G>A variant (valine substituted by isoleucine at position 417). They found that CBZ can inhibit efflux of the ABCC2 substrate 5,6-carboxyfluorescein diacetate in ABCC2 WT but only weakly in ABCC2 V417I transfected cells (Kim *et al.* 2010). In addition, ATPase activities in membrane vesicles obtained from the same cell lines were compared regarding their response to CBZ treatment. With increasing CBZ concentrations, ABCC2 WT vesicles exhibited substantial ATPase activity. In contrast, the variant only showed about 15 % of the maximum ABCC2 WT ATPase activity concluding that CBZ is a substrate of ABCC2 with impaired transporter activity in the variant (Kim *et al.* 2010). These data have to be interpreted with caution however, since neither assay measures the transport of CBZ directly. It is well established that drugs with inhibitory potencies may not necessarily be substrates themselves and ABCC2 has been suggested to have at least two binding sites with one modulatory and one transport binding site (Bodo *et al.* 2003, Zelcer *et al.* 2003). Furthermore, ATPase assays only measure the release of inorganic phosphate after stimulation of any ATPase activity but again are not a direct

transport measure. It is unclear why the CBZ-mediated inhibition of GS-MF efflux seen by Kim *et al.* could not be reproduced (Figure 2.3). However, consistent with the data presented here it has been reported that CBZ is not an inhibitor of the ABCC2 model substrate $E_2$17βG (Pedersen *et al.* 2008).

Three *ABCC2* polymorphisms have been extensively studied regarding their impact on CBZ or AED treatment response in epilepsy patients, namely rs717620 (-24C>T), rs2273697 (c.1249G>A), and rs3740066 (c.3972C>T). To follow-up on the negative CBZ *in vitro* studies, the clinical influence of these three SNPs on response to CBZ monotherapy was analysed using the well-accepted clinical outcome measures time to first seizure and time to 12-month remission. No statistically significant differences were found between genotypes after correcting for multiple testing (Figure 2.9).

To our knowledge, this is the first study analysing time to event data to explore the potential role of *ABCC2* polymorphisms on CBZ treatment efficacy. Previously published studies tested for differences between the genotypes of responders and non-responders using χ^2 or Fisher's exact test and/or logistic regression modelling. There are several advantages to survival analysis. First, censored data can be included and provide a powerful tool to make use of all available data whilst reducing any potential selection bias. Patients that do not respond well to treatment might be at a higher risk of dropout and therefore are at higher risk of being excluded from other methods compared with survival analysis. Second, there is no need to define a specific time-point in order to classify patients as responders or non-responders. Other advantages of the presented analysis include the prospective nature of data collection over a long period of time (6 years maximum) in a well-defined cohort of patients recruited into a randomised controlled trial. All selected patients were treated with CBZ monotherapy and most (> 90 %) had focal epilepsy.

This patient population does have some limitations, most notably the sample size. As the number of patients at risk drop over time the cumulative survival probabilities are not well described by Kaplan-Meier curves and parametric

models are difficult to fit. This is particularly an issue with the data after approximately 500 days (Figure 2.9). Up to 500 days, however, the survival probabilities are described well by parametric models based on the log-logistic distribution. Another limitation of the study could have been the dosing that was not dictated by the study protocol but rather was at the discretion of each investigator according to clinical guidelines (Marson *et al.* 2007). However, this approach was considered closer to clinical practice. The ethnic background of the patients was not taken into account for the analysis but the SANAD study was solely conducted in the UK and MAFs from all included patients correspond well to the MAFs obtained for Caucasians from HapMap (Table 2.2).

Several factors can account for the conflicting results previously reported. These include the sample size, definition of outcome, time of observation, epilepsy phenotype, co-treatment with other AEDs, prospective vs. retrospective studies, and differences in the study population. The survival analysis presented here is potentially the most unbiased study reported so far and is consistent with previous studies that failed to find any association between the investigated polymorphisms and CBZ treatment efficacy.

ABCC2, expressed in important biological barriers such as hepatocytes and proximale tubule epithelial kidney cells, is involved in the excretion of various conjugated endogenous and exogenous substrates (see chapter 1 section 1.2.2.2). Therefore, it is possible that a conjugated CBZ-metabolite is recognised as an ABCC2 substrate rather than the parent drug. Based on the presented *in vitro* data, this possibility cannot be excluded. The additional survival data, however, describe the efficacy of CBZ treatment *in vivo* and therefore include potential clinical effects derived from CBZ-metabolites for the outcomes and genotypes measured.

In conclusion, CBZ is not actively transported by ABCC2 *in vitro*. Clinically, no association between *ABCC2* polymorphisms rs717620 (-24C>T), rs2273697 (c.1249G>A), rs3740066 (c.3972C>T), and CBZ treatment response could be observed in patients from the SANAD study. This comprehensive analysis does not support a significant role for ABCC2 in CBZ treatment efficacy.

2.5. References

Arlanov, R.*, et al.* (2012) Functional characterization of protein variants of the human multidrug transporter ABCC2 by a novel targeted expression system in fibrosarcoma cells. *Human mutation* **33**, 750-762

Aronica, E.*, et al.* (2004) Expression and cellular distribution of multidrug resistance-related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. *Epilepsia* **45**, 441-451

Baltes, S.*, et al.* (2007) Differences in the transport of the antiepileptic drugs phenytoin, levetiracetam and carbamazepine by human and mouse P-glycoprotein. *Neuropharmacology* **52**, 333-346

Bodo, A.*, et al.* (2003) Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions. *The Journal of biological chemistry* **278**, 23529-23537

Brough, R., Papanastasiou, A. M., and Porter, A. C. (2007) Stringent and reproducible tetracycline-regulated transgene expression by site-specific insertion at chromosomal loci with pre-characterised induction characteristics. *BMC Mol Biol* **8**, 30

Colombo, F.*, et al.* (2013) A membrane vesicle-based assay to enable prediction of human biliary excretion. *Xenobiotica* **43**, 915-919

Dombrowski, S. M.*, et al.* (2001) Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. *Epilepsia* **42**, 1501-1506

Evers, R.*, et al.* (1998) Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *The Journal of clinical investigation* **101**, 1310-1319

Grover, S.*, et al.* (2012) Genetic association analysis of transporters identifies ABCC2 loci for seizure control in women with epilepsy on first-line antiepileptic drugs. *Pharmacogenet Genomics* **22**, 447-465

Grover, S., and Kukreti, R. (2013) A systematic review and meta-analysis of the role of ABCC2 variants on drug response in patients with epilepsy. *Epilepsia*

Heredi-Szabo, K.*, et al.* (2008) Characterization of 5(6)-carboxy-2,'7'-dichlorofluorescein transport by MRP2 and utilization of this substrate as a fluorescent surrogate for LTC4. *Journal of biomolecular screening* **13**, 295-301

Hilger, E.*, et al.* (2012) Lack of association between ABCC2 gene variants and treatment response in epilepsy. *Pharmacogenomics* **13**, 185-190

Keppler, D., Jedlitschky, G., and Leier, I. (1998) Transport function and substrate specificity of multidrug resistance protein. *Methods in enzymology* **292**, 607-616

Kidron, H.*, et al.* (2012) Impact of probe compound in MRP2 vesicular transport assays. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences* **46**, 100-105

Kim, D. W.*, et al.* (2009) Lack of association between ABCB1, ABCG2, and ABCC2 genetic polymorphisms and multidrug resistance in partial epilepsy. *Epilepsy research* **84**, 86-90

Kim, W. J.*, et al.* (2010) A nonsynonymous variation in MRP2/ABCC2 is associated with neurological adverse drug reactions of carbamazepine in patients with epilepsy. *Pharmacogenet Genomics* **20**, 249-256

Kleinbaum, D. G., and Klein, M. (2012) Survival analysis: A self-learning text / David G. Kleinbaum, Mitchel Klein,*Statistics for biology and health*, *New York, NY : Springer, c2012.*
3rd ed.

Kubota, H.*, et al.* (2006) Distribution and functional activity of P-glycoprotein and multidrug resistance-associated proteins in human brain microvascular endothelial cells in hippocampal sclerosis. *Epilepsy Res.* **68**, 213-228

Kwan, P.*, et al.* (2011) Gene-wide tagging study of the association between ABCC2, ABCC5 and ABCG2 genetic polymorphisms and multidrug resistance in epilepsy. *Pharmacogenomics* **12**, 319-325

Lechner, C.*, et al.* (2010) Development of a fluorescence-based assay for drug interactions with human Multidrug Resistance Related Protein (MRP2; ABCC2) in MDCKII-MRP2 membrane vesicles. *Eur J Pharm Biopharm* **75**, 284-290

Lehmann, T.*, et al.* (2001) Expression of MRP1 and related transporters in human lung cells in culture. *Toxicology* **167**, 59-72

Leier, I.*, et al.* (1994) Characterization of the ATP-dependent leukotriene C4 export carrier in mastocytoma cells. *European journal of biochemistry / FEBS* **220**, 599-606

Luna-Tortos, C., Fedrowitz, M., and Loscher, W. (2010) Evaluation of transport of common antiepileptic drugs by human multidrug resistance-associated proteins (MRP1, 2 and 5) that are overexpressed in pharmacoresistant epilepsy. *Neuropharmacology* **58**, 1019-1032

Luna-Tortos, C.*, et al.* (2009) The antiepileptic drug topiramate is a substrate for human P-glycoprotein but not multidrug resistance proteins. *Pharm.Res.* **26**, 2464-2470

Ma, C. L.*, et al.* (2014) Association of SCN1A, SCN2A and ABCC2 gene polymorphisms with the response to antiepileptic drugs in Chinese Han patients with epilepsy. *Pharmacogenomics* **15**, 1323-1336

Marson, A. G.*, et al.* (2007) The SANAD study of effectiveness of carbamazepine, gabapentin, lamotrigine, oxcarbazepine, or topiramate for treatment of partial epilepsy: an unblinded randomised controlled trial. *Lancet* **369**, 1000-1015

Pedersen, J. M.*, et al.* (2008) Prediction and identification of drug interactions with the human ATP-binding cassette transporter multidrug-resistance associated protein 2 (MRP2; ABCC2). *J Med Chem* **51**, 3275-3287

Pratt, S.*, et al.* (2006) Kinetic validation of the use of carboxydichlorofluorescein as a drug surrogate for MRP5-mediated transport. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences* **27**, 524-532

Qu, J.*, et al.* (2012) ABCC2 Polymorphisms and Haplotype are Associated with Drug Resistance in Chinese Epileptic Patients. *CNS Neurosci Ther*

Radisch, S.*, et al.* (2014) A comprehensive functional and clinical analysis of ABCC2 and its impact on treatment response to carbamazepine. *Pharmacogenomics J*

Roelofsen, H.*, et al.* (1997) Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells. *Gastroenterology* **112**, 511-521

Seo, T.*, et al.* (2008) ABCC2 haplotype is not associated with drug-resistant epilepsy. *The Journal of pharmacy and pharmacology* **60**, 631-635

Smith, P. K.*, et al.* (1985) Measurement of protein using bicinchoninic acid. *Analytical biochemistry* **150**, 76-85

Speed, D.*, et al.* (2013) A genome-wide association study and biological pathway analysis of epilepsy prognosis in a prospective cohort of newly treated epilepsy. *Human molecular genetics*

Sporis, D.*, et al.* (2013) Lack of association between polymorphism in ABCC2 gene and response to antiepileptic drug treatment in Croatian patients with epilepsy. *Collegium antropologicum* **37**, 41-45

Ufer, M.*, et al.* (2009) Non-response to antiepileptic pharmacotherapy is associated with the ABCC2 -24C>T polymorphism in young and adult patients with epilepsy. *Pharmacogenet Genomics* **19**, 353-362

Ufer, M.*, et al.* (2011) Impact of ABCC2 genotype on antiepileptic drug response in Caucasian patients with childhood epilepsy. *Pharmacogenet.Genomics* **21**, 624-630

Zelcer, N.*, et al.* (2003) Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP binding cassette C2). *The Journal of biological chemistry* **278**, 23538-23544

Chapter 3

A screening approach of antiepileptic drugs as potential drug transporter substrates

Contents

3.1. Introduction

Membrane transporters are beginning to be recognised as important components in drug ADME and the FDA recommends an increasing number of transporters to be included in drug interaction studies in clinical development (see chapter 1 section 1.2). Despite their emerging role, drug transport processes have rarely been studied for already licensed AEDs and the mechanism of BBB translocation is largely unknown or considered to rely on passive diffusion. The available studies have largely focused on ABCB1 (MDR1, Pgp), ABCC1 (MRP1), ABCC2 (MRP2), ABCC5 (MRP5), and ABCG2 (BCRP) as potential mediators for pharmacoresistance, but results have mostly been conflicting or negative (see chapter 1 sections 1.3.3 and 1.4). In view of the fact that the human genome encodes for at least 390 SLC transporters (see chapter 1 section 1.2.1) and 48 ABC transporters (see chapter 1 section 1.2.2), more research is needed to clarify the potential role drug transporters play in the uptake and efflux of established AEDs, particularly at the BBB.

A better understanding of AED ADME will be important to make AED treatment more effective and safer. To address this research need, a screening approach was applied in order to find and characterise unknown transport processes for six major AEDs, namely CBZ, LTG, TPM, LEV, VPA, and PHT (see chapter 1 section 1.3.3).

3.2. Materials and methods

3.2.1. Materials

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich Company Ltd., Gillingham, UK. EBM-2 basal medium was obtained from Lonza Group Ltd., Basel, Switzerland. TaqMan® gene expression assays, TaqMan® gene expression master mix, TaqMan® reverse transcription reagents, collagen I from rat tail, fibroblast growth factor-basic (amino acids 1-155) recombinant human protein (bFGF), chemically defined lipid concentrate, and *SLC7A5* targeting/negative control #1 Silencer® Select siRNAs came from Life Technologies Ltd., Paisley, UK. Tritium-labelled CBZ ([^{3}H]-CBZ, 1 mCi/ml, specific activity 10.0 Ci/mmol), tritium-labelled LTG ([^{3}H]-LTG, 1 mCi/ml, specific activity 5.0 Ci/mmol), tritium-labelled TPM ([^{3}H]-TPM, 1 mCi/ml, specific activity 8.0 Ci/mmol), tritium-labelled LEV ([^{3}H]-LEV, 1 mCi/ml, specific activity 5.0 Ci/mmol), and tritium-labelled GBP ([^{3}H]-GBP, 1 mCi/ml, specific activity 1.0 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc., St. Louis, MO, USA. Tritium-labelled VPA ([^{3}H]-VPA, 1 mCi/ml, specific activity 55.0 Ci/mmol) and tritium-labelled PHT ([^{3}H]-PHT, 1 mCi/ml, specific activity 1.1 Ci/mmol) came from Moravek Biochemicals Inc., Brea, CA, USA and tritium-labelled digoxin ([^{3}H]-digoxin, 1 mCi/ml, specific activity 40.0 Ci/mmol) from PerkinElmer, Seer Green, UK. Gold Star scintillation cocktail was purchased from Meridian Biotechnologies Ltd., Epsom, UK, MK571, methotrexate, and Ko143 from Tocris Bioscience, Bristol, UK, and montelukast from Insight Biotechnology Ltd., Wembley, UK. DharmaFECT1 transfection reagent, Dharmacon targeting siGENOME siRNAs - SMARTpool, and non-targeting siGENOME siRNA pool #2 were obtained from Fermentas GmbH – Thermo Fisher Scientific, St. Leon-Rot, Germany. FlexiTube GeneSolution for human *ABCC5* and AllStars negative control siRNA came from Qiagen Ltd., Manchester, UK.

3.2.2. Cell lines and culture conditions

3.2.2.1. hCMEC/D3 cell line

The human microvascular brain endothelial cell line hCMEC/D3 is an immortalised cell clone derived from primary brain endothelial cells from a female epilepsy patient who underwent temporal lobectomy (Weksler *et al.* 2005). The primary cells were immortalised using a lentiviral transduction approach with human telomerase reverse transcriptase and the simian vacuolating virus 40 large T antigen (Weksler *et al.* 2005). hCMEC/D3 cells were cultured at 37 °C and 5 % CO_2 in EBM-2 medium supplemented with 5 % FBS (v/v), 1 % penicillin-streptomycin (v/v), 10 mM HEPES, 1.4 µM hydrocortisone, 5 µg/ml ascorbic acid, 1 % chemically defined lipid concentrate (v/v), and 1 ng/ml bFGF. hCMEC/D3 are adhesive cells and were cultured on collagen-coated plates or flasks. For collagen-coating, collagen I from rat tail was diluted with distilled water to give a final protein concentration of 60 µg/ml. Plates/flasks were treated with the solution for 1-2 hours at 37 °C. After treatment, the collagen solution was removed and plates/flasks washed once with HBSS.

3.2.3. Distribution coefficient (logD, pH 7.4)

The distribution coefficient (logD, pH 7.4) is a commonly used measure for the lipophilicity of a drug. It is related to the partitioning coefficient (logP) and describes the distribution of a drug in a biphasic system at equilibrium, usually 1-octanol and water (Kenakin 2012). While the logP does not take account of the potentially ionised fraction of a drug, the logD is the distribution at a given pH and therefore independent of the compounds net charge (Kenakin 2012).

The logD was determined at a physiological pH of 7.4 using transport buffer as aqueous phase (HBSS supplemented with 25 mM HEPES, adjusted to pH 7.4) and 1-octanol as organic phase (Davies *et al.* 2009, Dickens *et al.* 2013a, Yunger *et al.* 1981). 0.64 µl from a 1/10 dilution of tritium-labelled drug was mixed with 4 ml transport buffer and equally divided into 4x1 ml fractions, each supplemented

with 1 ml 1-octanol. The biphasic solutions were vigorously shaken for 15 minutes and subsequently centrifuged at 250 g for 5 minutes at room temperature (Heraeus™ Megafuge™ 11R centrifuge with T41 swing-out rotor, Thermo Fisher Scientific, Leicestershire, UK). 500 µl from both phases were transferred into scintillation tubes containing 4 ml scintillation cocktail. Radioactivity was measured by means of scintillation counting (1500 Tri Carb LS Counter, Packard, now PerkinElmer, Seer Green, UK) and results obtained as disintegrations per minute (DPM). The logD was calculated as follows: $logD\ (pH\ 7.4) = \log\left(\frac{DPM\ (1-octanol\ phase)}{DPM\ (transport\ buffer\ phase)}\right)$

3.2.4. Drug accumulation assays

Accumulation assays were prepared by seeding 300,000 hCMEC/D3 cells/well onto Nunc™ 6-well plates (Fisher Scientific UK Ltd., Loughborough, UK) with culturing for two days to reach confluence. Confluent cells were then washed two times with pre-warmed (37 °C) HBSS and the assay initiated by adding 1 ml/well pre-warmed master mix. A separate master mix was prepared for each condition to be tested, consisting of transport buffer (HBSS supplemented with 25 mM HEPES, adjusted to pH 7.4), 0.6 µCi/ml tritium-labelled "hot" drug (except [^{3}H]-PHT which was 0.3 µCi/ml), 5 µM non-labelled corresponding "cold" drug, and, if applicable, the inhibitor or vehicle only as control. The total vehicle concentration did not exceed 1.1 % per reaction (dimethyl sulfoxide (DMSO) and methanol). The assay was incubated at 37 °C or 4 °C for the desired time and subsequently washed with 1 ml ice-cold HEPES. Washing was repeated three times and the cells finally lysed in 400 µl 10 % SDS. Each lysate was transferred into a scintillation tube containing 4 ml scintillation cocktail and radioactivity determined by scintillation counting (1500 Tri Carb LS Counter, Packard, now PerkinElmer, Seer Green, UK).

3.2.5. siRNA transfection

Gene silencing or RNA interference (RNAi) experiments by means of small interfering RNA (siRNA) transfections were essentially carried out as recommended

by the Dharmacon siGENOME siRNA manufacturer Fermentas GmbH – Thermo Fisher Scientific, St. Leon-Rot, Germany. The siRNAs for all genes to be investigated were from the Dharmacon siGENOME SMARTpool product line from Thermo Fisher, each with four different targeting sequences pooled for higher knockdown efficiency. Transfection with the non-targeting siGENOME siRNA pool #2 was utilised as negative control. The functional positive control targeting *SLC7A5* and corresponding non-targeting control #1 were from the Silencer® Select product line from Life Technologies with one siRNA sequence only. For *ABCC5*, an additional set of four pooled siRNAs plus the corresponding non-targeting control (AllStars) was obtained from the FlexiTube GeneSolution line from Qiagen. All siRNA sequences and corresponding identification numbers are listed in the appendix. Independent from manufacturer, gene silencing assays were carried out using the same protocol with DharmaFECT1 as transfection reagent.

100,000 hCMEC/D3 cells/well (or as specified) were seeded onto collagen-coated Nunc™ 6-well plates and incubated for 24 hours to allow attachment to the surface. The growth medium (3.2.2.1) was slightly modified and did not contain penicillin-streptomycin throughout the experiments. For transfection, a 10x concentrated siRNA solution (1/10 of final volume) and 10x concentrated DharmaFECT1 solution (1/10 of final volume) was prepared separately in serum-free EBM-2 medium (no supplements). After assay optimisation, the final DharmaFECT1 concentration used was 0.2 % (or as specified) and the final siRNA concentrations 25 nM (or as specified). Both solutions were gently mixed and incubated for 5 minutes at room temperature before they were combined, gently mixed, and again incubated for 20 minutes at room temperature. Finally, the combined DharmaFECT1/siRNA solutions were filled-up with growth medium to give the final volume of 2 ml/well on a 6-well plate (1.6 ml culture medium to be added). The old culture medium was replaced by the transfection solution and the cells incubated at 37 °C for 48 hours. Downstream experiments included gene expression (3.2.6) and drug accumulation assays (3.2.4).

3.2.6. Gene expression

3.2.6.1. RNA extraction

RNA extraction was carried out using a standard phenol-chloroform extraction method based on the TRIzol® Reagent protocol from Life Technologies Ltd., Paisley, UK. 1 ml/well Tri Reagent® was used for cell homogenisation on 6-well plates and the homogenate incubated for 5 minutes at room temperature. 0.2 ml chloroform was added to each sample, vigorously shaken by hand for 15 seconds, and again incubated at room temperature for 3 minutes. Centrifugation (Heraeus™ Fresco™ 21 centrifuge with 24 x 1.5/2.0 ml rotor, Thermo Fisher Scientific, Leicestershire, UK) at 12,000 g for 15 minutes at 4 °C resulted in a phase separation with three layers. The top layer with RNA was further processed and the RNA precipitated by adding 0.5 ml 100 % isopropanol. Each sample was incubated for 10 minutes at room temperature and centrifuged at 12,000 g at 4 °C for another 10 minutes (Heraeus™ Fresco™ 21 centrifuge with 24 x 1.5/2.0 ml rotor, Thermo Fisher Scientific, Leicestershire, UK). The supernatant was removed and the precipitated RNA washed with 1 ml 70 % ethanol. A final centrifugation step at 7,500 g for 5 minutes at 4 °C (Heraeus™ Fresco™ 21 centrifuge with 24 x 1.5/2.0 ml rotor, Thermo Fisher Scientific, Leicestershire, UK) allowed the ethanol to be removed. The RNA pellet was dried for 10 minutes at room temperature and resuspended in RNAse-free, molecular biology-grade water. RNA concentrations and purity were analysed on a NanoDrop™ 8000 spectrophotometer (Fisher Scientific UK Ltd., Loughborough, UK).

3.2.6.2. RT-PCR

To analyse gene expression, messenger RNA (mRNA) was first reverse-transcribed into copy DNA (cDNA) using the TaqMan® reverse transcription reagents for reverse transcriptase (RT)-polymerase chain reaction (PCR) according to the manufacturer. Each reaction mix contained 1 µl 10x TaqMan® RT buffer (1x final), 2.2 µl 25 mM $MgCl_2$ (5.5 mM final), 2 µl 2.5 mM deoxyribonucleoside triphosphates (dNTPs) mixture (500 µM final), 0.5 µl 50 µM random hexamers (2.5 µM final), 0.2 µl of

20 U/µl RNAse inhibitor (0.4 U/µl final), 0.35 µl of 50 U/µl MultiScribe reverse transcriptase (1.25 U/µl final), 0.4 µg RNA, and RNAse-free, molecular biology-grade water to give a final volume of 10 µl. The reaction mix was incubated in a Veriti® 96-well thermal cycler (Life Technologies Ltd., Paisley, UK) running the following programme:

1. 25 °C for 10 minutes
2. 48 °C for 30 minutes
3. 95 °C for 5 minutes
4. Hold 4 °C

Single-stranded cDNA concentrations and purity were analysed on a NanoDrop™ 8000 spectrophotometer (Fisher Scientific UK Ltd., Loughborough, UK) and each sample diluted to a concentration of 50 ng/µl. The cDNA was stored at -20 °C until needed.

3.2.6.3. Real-time PCR

The relative gene expression (delta threshold cycle (ΔC_t) method) was determined on an Applied Biosystems® 7900HT fast real-time PCR system using a 384-well block (Life Technologies Ltd., Paisley, UK). TaqMan® gene expression assays were ordered for each gene of interest (*GoI*), consisting of two unlabelled primers and a gene-specific probe that is covalently attached to the fluorescent reporter dye FAM™. Gene expression was normalised to the human housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) by subtracting the C_t of each gene from the C_t obtained for *GAPDH* expression (ΔC_t). The software calculated the optimal threshold and baseline levels automatically. In contrast to FAM™-labelled *GoI* gene expression assays, the *GAPDH* gene expression assay consisted of a VIC®-labelled reporter dye and was primer limited, allowing multiplex reactions. Multiplex reactions reduce the number of required assays and give more robust results due to the minimised influence of other factors such as pipetting. TaqMan® assay probe sequences and identification numbers are listed in the appendix. Each multiplex reaction consisted of 10 µl 2x TaqMan® master mix (1x final), 1 µl *GoI* TaqMan® gene expression assay, 1 µl *GAPDH* TaqMan® gene expression assay, 2 µl cDNA

(50 ng/µl), and 6 µl molecular biology-grade water. The following program was used for real-time amplification:

1. 95 °C for 15 minutes
2. 95 °C for 15 seconds
3. 60 °C for 60 seconds
4. Repeat cycles 2 and 3, 50x

3.2.7. Statistical analysis

All data are presented as means ± SD. A one-way ANOVA followed by a Dunnett's post-hoc test was carried out for statistical analysis of all experiments with multiple comparisons. Single comparisons were analysed by an independent, two-tailed t-test. Significant results are indicated with * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$. Analysis was performed with SPSS® Statistics version 20 (IBM United Kingdom Ltd., Hampshire, UK).

3.3. Results

3.3.1. Lipophilicity and cellular accumulation of AEDs

Brain uptake of AEDs could, theoretically, solely or dominantly occur by passive-transcellular diffusion across the BBB. The lipophilicity of a drug is a strong and important predictor for passive diffusion (see chapter 1 section 1.1.2) and the logD of six major AEDs, namely CBZ, LTG, TPM, LEV, VPA, and PHT, was determined at a physiological pH of 7.4 (Table 3.1). In addition, the predicted logD (pH 7.4) was derived from www.chemspider.com using the ACD labs algorithm and compared to the experimentally obtained logD (pH 7.4).

Except for TPM, the predicted and experimentally derived logDs (pH 7.4) were of similar order of magnitude and resulted in the same drug rank order (Table 3.1). A huge discrepancy was observed for TPM with a predicted logD (pH 7.4) of 2.97 and an experimentally derived logD (pH 7.4) of -0.64. In the literature, an experimental logD (pH 7.4) of 0.51 was reported (Maryanoff *et al.* 1998). Given that TPM is a sulfamate-substituted monosaccharide (Janssen Pharmaceuticals Inc. 2012) with a predicted high polar surface area of 123.92 $Å^2$ (van de Waterbeemd *et al.* 1998) (www.chemspider.com), the predicted logD (pH 7.4) of 2.97 seems very unlikely and the results presented here and by Maryanoff *et al*. are substantially lower.

To correlate the lipophilicity of each drug to cellular uptake, drug accumulation was assessed in the hCMEC/D3 cell line as a BBB model after 30 minutes at 37 °C (Figure 3.1 A) and compared to the experimentally derived logDs (pH 7.4). No notable correlation could be observed and linear regression yielded an R^2 of only 0.02 with a p-value of 0.81 (Figure 3.1 B). The null hypothesis was that the slope of the regression line is equal to zero. Due to the fact that LTG is a known substrate of SLC22A1 (OCT1) (Dickens *et al.* 2012), the same analysis was repeated but excluding LTG. The linear correlation markedly improved from an R^2 of 0.02 to 0.3 (Figure 3.1 C), showing the substantial influence of drug transporters on this analysis. However, the p-value remained non-significant on the 5 % level with

p = 0.37, suggesting that additional drug transporters may be involved in AED accumulation.

This comparison can only be considered as a first step and has to be interpreted with care since intracellular accumulation was only measured at one time point using a single concentration. Other factors such as intracellular protein binding might have affected total drug uptake in addition to lipophilicity and transporters. However, because passive diffusion is a strong predictor for drug uptake in the absence of transporters, a stronger and significant linear correlation would have been expected.

Table 3.1: Lipophilicity of six antiepileptic drugs

	Predictive logD (pH 7.4)	Experimental logD (pH 7.4)
Phenytoin	2.49	1.95±0.02
Carbamazepine	1.90	1.60±0.01
Valproate	0.16	0.04±0.05
Lamotrigine	-0.19	0.00±0.01
Levetiracetam	-0.67	-0.60±0.01
Topiramate	2.97	-0.64±0.01

n = 3 independent experiments each in quadruplicate

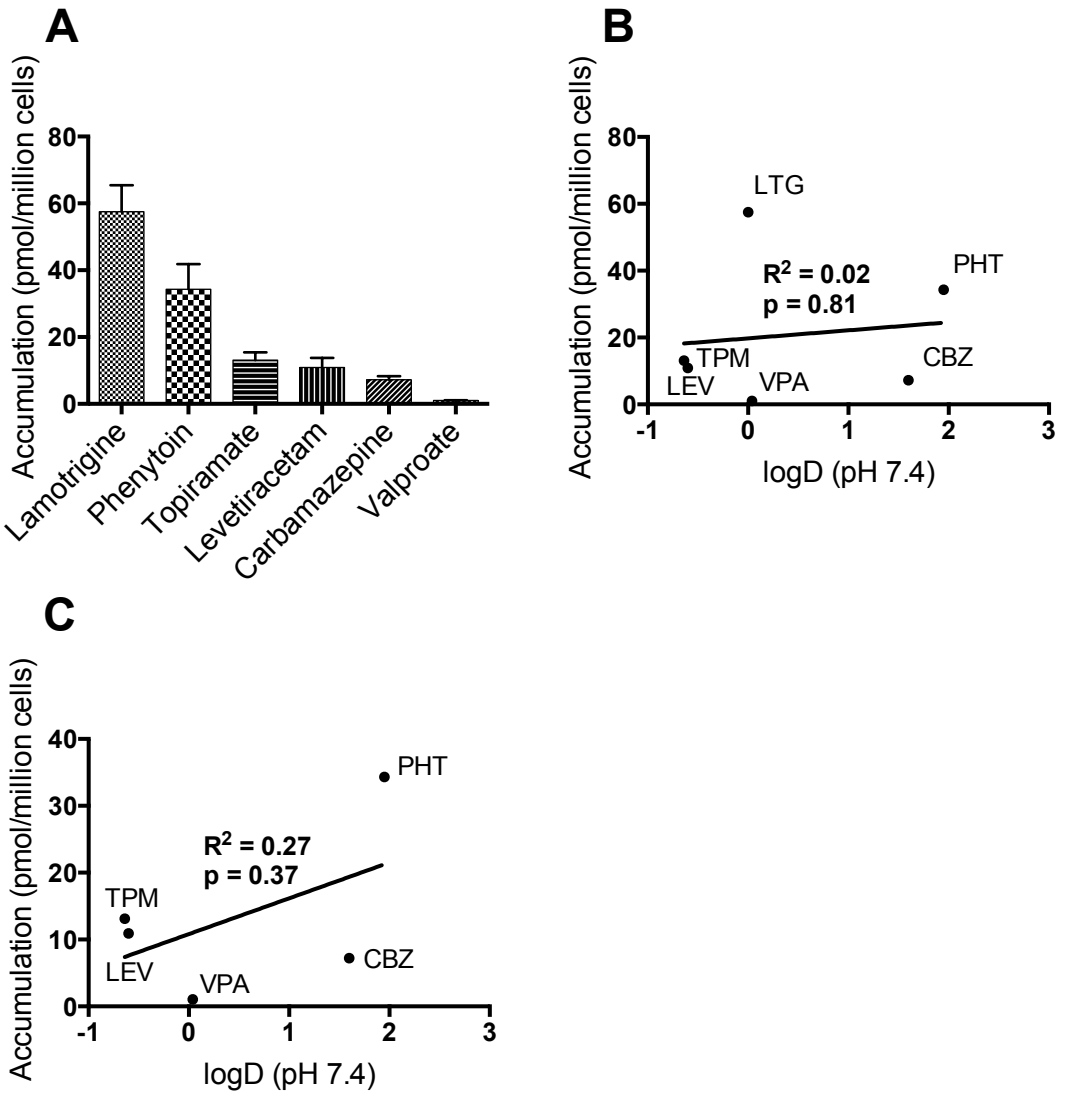


Figure 3.1: Accumulation of six AEDs in the hCMEC/D3 cell line and correlation with lipophilicity

A) Accumulation of six antiepileptic drugs (AEDs, 10 µM each) in hCMEC/D3 cells after 30 minutes at 37 °C. Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate); B) Lipophilicity of six AEDs, given as logD at pH 7.4, plotted against the mean accumulation in hCMEC/D3 cells as indicated in A; C) Lipophilicity of five AEDs (without LTG), given as logD at pH 7.4, plotted against the mean accumulation in hCMEC/D3 cells as indicated in A; LTG = lamotrigine, PHT = phenytoin, TPM = topiramate, LEV = levetiracetam, CBZ = carbamazepine, VPA = valproate

3.3.2. Initial screening of antiepileptic drug transport in the hCMEC/D3 cell line using low molecular weight inhibitors

Drug accumulation in the hCMEC/D3 cell line and the logD (pH 7.4) did not correlate to a statistically significant degree. Therefore, other mechanisms such as drug transporters may be involved in AED uptake and/or efflux. A screening approach utilising known chemical drug transporter inhibitors (including substrates as competitive inhibitors) was applied to obtain information about potentially involved drug transporters.

The selected compounds are listed in Table 3.2 with corresponding typical and important drug transporter classes affected. The term “class” shall denote a drug transporter family, subfamily, or further sub-divisions such as the OATs or OCTs within the SLC22A subfamily (see chapter 1 section 1.2.1.3). Chemical inhibitors can never be considered as absolutely specific and thus a transporter class was regarded as being affected when one or more transporters have been shown to interact with the designated compound. This should not imply, however, that every member from within these transporter classes is necessarily affected. The accumulation of all six AEDs was determined in hCMEC/D3 cells after 30 minutes at 37 °C in the presence of various chemical inhibitors and compared to the control accumulation with solvent only (Figure 3.2).

Overall, most of the inhibitors tested did not result in an altered AED accumulation, except for PHT. PHT showed a significantly reduced accumulation in the presence of several inhibitors, namely MK571, verapamil, phloretin, and naringenin, suggesting one or more transporters being involved in PHT uptake. VPA accumulation, by contrast, was not affected by any inhibitor and notably very close to background level. CBZ accumulation was also not affected by any of the inhibitors tested. LTG accumulation was lower in the presence of verapamil, consistent with the results obtained by Dickens *et al.* showing that LTG is a substrate for SLC22A1 (OCT1) (Dickens *et al.* 2012). Except for a slightly reduced

accumulation in the presence of phloretin, possibly also a result of SLC22A1 inhibition, no other inhibitor tested had an effect on LTG accumulation. Interestingly, the accumulation of TPM and LEV was enhanced in the presence of MK571, a leukotriene D_4 receptor antagonist and prototypical ABCC transporter inhibitor. On average, TPM accumulation was enhanced by 53 % and LEV accumulation by 23 %. None of the other tested inhibitors had an effect.

Table 3.2: Selected low molecular weight chemical drug transporter inhibitors/substrates for the initial screening of antiepileptic drug transport in the hCMEC/D3 cell line

Compound	Concentration	Affected drug transporter classes	References
MK571	50 µM	ABCCs, OATPs	(Keppler 2011, Letschert *et al.* 2005)
Probenecid	500 µM	OATs	(Burckhardt *et al.* 2011)
Verapamil	100 µM	ABCBs, ABCGs, OCTs, OCTNs	(Giacomini *et al.* 2010, Matsson *et al.* 2009, Nies *et al.* 2011, Ohashi *et al.* 1999, Yabuuchi *et al.* 1999)
Methotrexate	100 µM	OATPs, OATs, ABCCs, ABCGs	(Badagnani *et al.* 2006, Burckhardt *et al.* 2011, Chen *et al.* 2003, Keppler 2011)
Phloretin	55 µM	ABCGs, ABCBs	(Zhang *et al.* 2003, Zhang *et al.* 2004)
Naringenin	100 µM	ABCGs, ABCBs, (OATPs)	(Bailey *et al.* 2007, Dresser *et al.* 2002, Shirasaka *et al.* 2009, Zhang *et al.* 2004)

The flavonoid naringenin has been suggested to act as inhibitor of OATP transporters but direct evidence for human OATPs is currently lacking

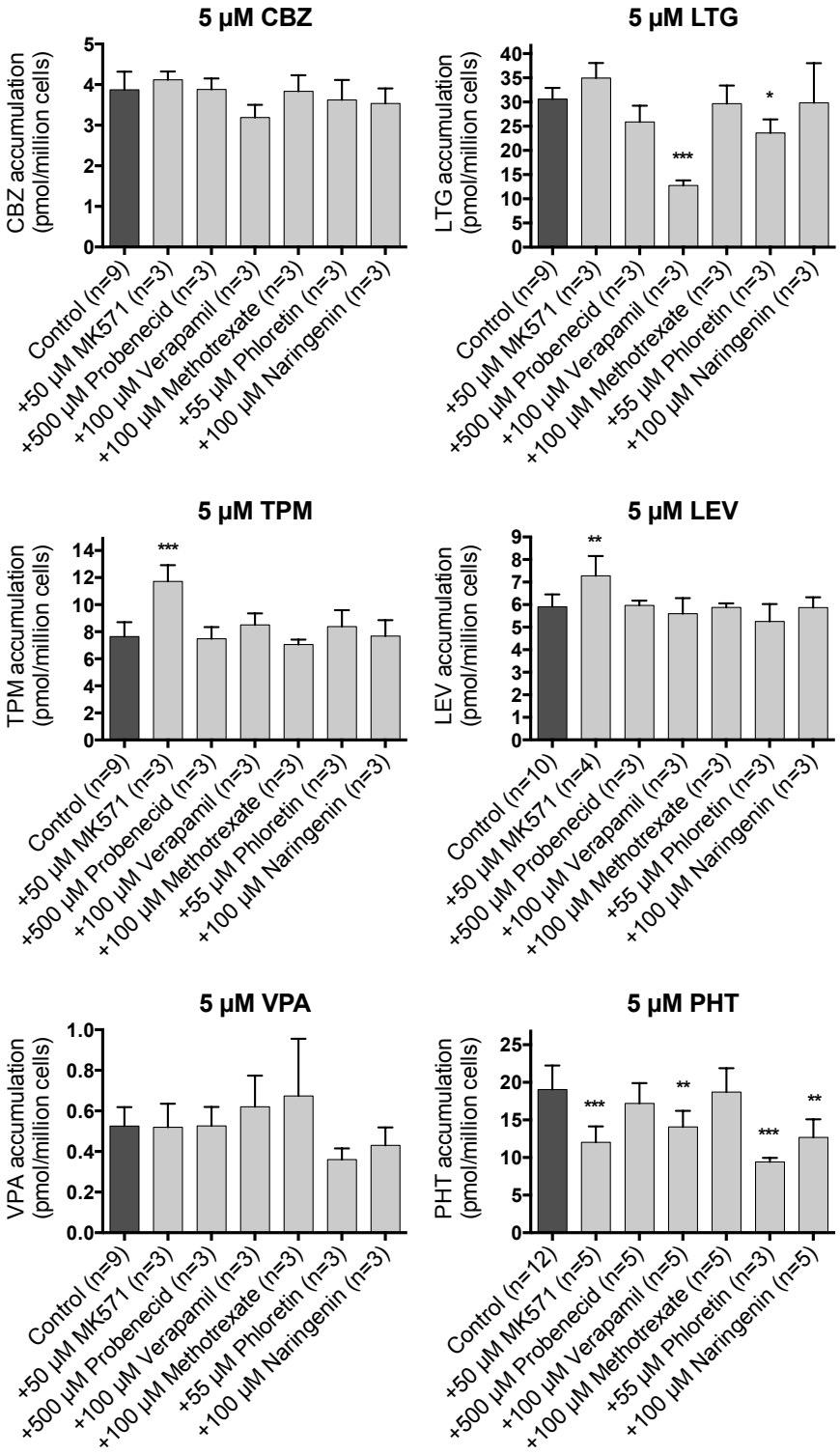


Figure 3.2: Initial screening of antiepileptic drug transport in the hCMEC/D3 cell line using low molecular weight inhibitors

Accumulation of six antiepileptic drugs in the presence of different known drug transporter inhibitors in the hCMEC/D3 cell line after 30 minutes at 37 °C. Data are expressed as means ± standard deviation (n = 3-12 independent experiments in triplicate). Significant results are indicated with * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. CBZ = carbamazepine, LTG = lamotrigine, TPM = topiramate, LEV = levetiracetam, VPA = valproic acid, PHT = phenytoin

3.3.3. Second inhibitor screening to further assess topiramate and phenytoin transport in the hCMEC/D3 cell line

The results from the initial screening approach indicate TPM and PHT as the most promising AEDs to be further investigated as potential drug transporter substrates in the hCMEC/D3 cell line. An unrecognised efflux transporter or transporters may be involved in TPM transport while an uptake transporter or transporters seem to mediate PHT uptake.

To gain additional information about the potentially involved drug transporter classes, a second screening was applied for these two drugs utilising a different set of chemical inhibitors. Because TPM accumulation is enhanced in the presence of the prototypical ABCC transporter inhibitor MK571, additional ABCC transporter inhibitors were included into this set. As for the first screening approach, the selected inhibitors are summarised in Table 3.3 with the corresponding typical and important drug transporter classes affected. MK571 and phloretin were included as positive controls, respectively.

The second screening resulted in two additional positive hits affecting TPM accumulation, montelukast and prazosin (Figure 3.3). Montelukast, another leukotriene D_4 receptor antagonist, is a typical ABCC transporter inhibitor showing the same enhanced TPM uptake as was observed with MK571. Prazosin resulted in a decrease in accumulation, suggesting that in addition to efflux, TPM influx might also be carrier-mediated. For PHT, additional positive results were obtained with montelukast, prazosin, and quercetin, all lowering PHT cell accumulation (Figure 3.3). In conclusion from both screenings, TPM could potentially be a substrate for an efflux transporter from the ABCC subfamily. Given that all the tested flavonoids (naringenin, phloretin, quercetin) and leukotriene D_4 receptor antagonists (MK571, montelukast) resulted in reduced PHT accumulation, a SLCO (OATP) transporter might be involved in PHT uptake.

Table 3.3: Selected chemical drug transporter inhibitors for second screening to further assess topiramate and phenytoin transport in the hCMEC/D3 cell line

Compound	Concentration	Affected drug transporter classes	References
Montelukast	50 µM	ABCCs, OATPs	(Letschert *et al.* 2006, Roy *et al.* 2009)
Diclofenac	50 µM	ABCCs, OATs, OATPs, OCTs, ABCGs	(Burckhardt *et al.* 2011, Khamdang *et al.* 2002, Kindla *et al.* 2011, Lagas *et al.* 2009, Reid *et al.* 2003)
Indomethacin	100 µM	ABCCs, OATs, OCTs	(Burckhardt *et al.* 2011, Khamdang *et al.* 2002, Reid *et al.* 2003)
Prazosin	100 µM	OCTs, ABCGs	(Matsson *et al.* 2007, Matsson *et al.* 2009, Nies *et al.* 2011)
Ko143	1 µM	ABCGs	(Allen *et al.* 2002)
Quercetin	50 µM	OATPs, ABCGs, ABCBs, ABCCs	(Mandery *et al.* 2010, Matsson *et al.* 2009, Wu *et al.* 2012, Zhang *et al.* 2004)
Mycophenolic Acid	50 µM	OATs	(Uwai *et al.* 2007, Wolff *et al.* 2007)
Thyroxine (T4)	1 µM	OATPs	(Roth *et al.* 2011)

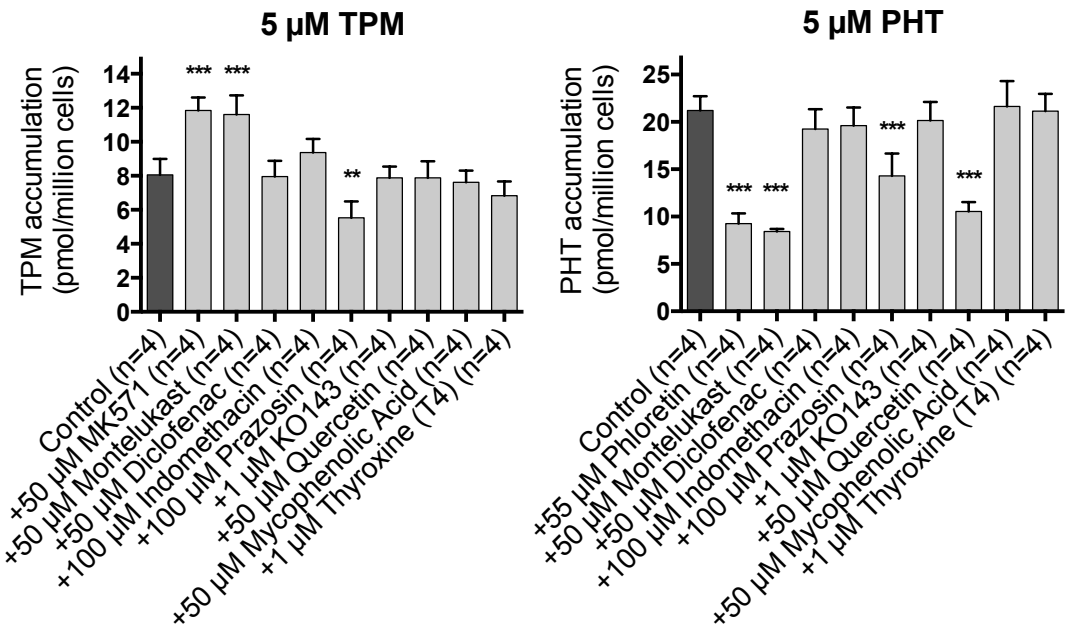


Figure 3.3: Second inhibitor screening to further assess topiramate and phenytoin transport in the hCMEC/D3 cell line

Accumulation of topiramate and phenytoin in the presence of various drug transporter inhibitors in the hCMEC/D3 cell line after 30 minutes at 37 °C. Data are expressed as means ± standard deviation (n = 4 independent experiments in triplicate). Significant results are indicated with ** for $p<0.01$ and *** for $p<0.001$. TPM = topiramate, PHT = phenytoin

TPM has previously been described as a weak substrate for ABCB1 (MDR1, Pgp) (Luna-Tortos *et al.* 2009) and although the typical ABCB1 inhibitor verapamil had no effect on TPM accumulation, ABCB1 transport cannot be fully excluded as the underlying reason for the results obtained. To gain more confidence that impaired ABCB1 transport is not involved in the observed increase in TPM accumulation, an additional experiment was carried out with the ABCB1 substrate digoxin as a positive control (Giacomini *et al.* 2010). The accumulation of 5 µM TPM and 5 µM digoxin was determined after 30 minutes in hCMEC/D3 cells in the presence of two relatively specific ABCB1 inhibitors, tariquidar and PSC-833 (Dickens *et al.* 2013b), as well as the typical ABCC inhibitor MK571. While the accumulation of digoxin increased in the presence of all three inhibitors tested, TPM accumulation only increased in the presence of MK571 (Figure 3.4). MK571 is

considered a prototypical ABCC transporter inhibitor but has also been reported to inhibit ABCB1, explaining the positive results obtained with digoxin (Matsson *et al.* 2009). These results demonstrate that the enhanced TPM accumulation observed in the presence of MK571 is not a result of an impaired ABCB1 transport but other, so far unknown, transport process.

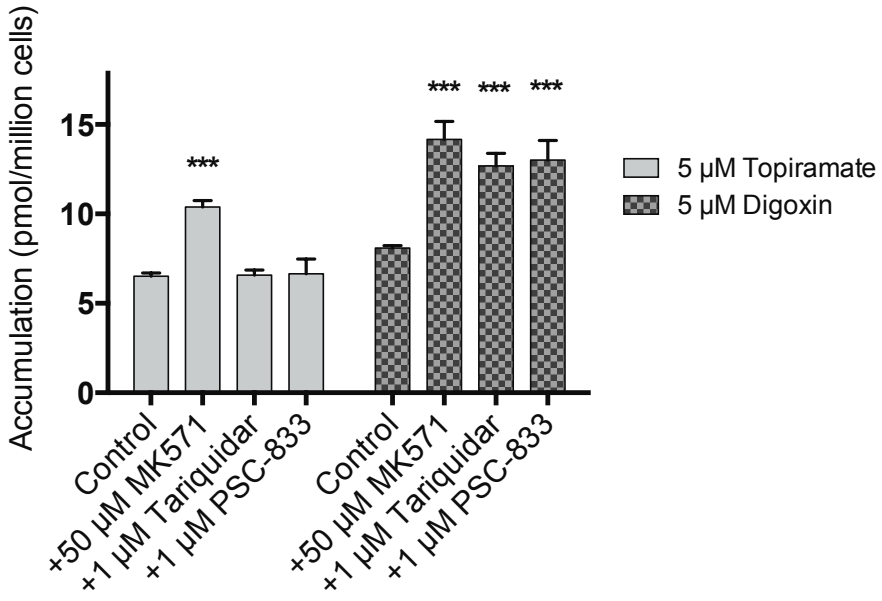


Figure 3.4: Investigation of ABCB1 (MDR1, Pgp) activity on topiramate accumulation

The accumulation of topiramate and digoxin (positive control) was tested in the presence of the two ABCB1 inhibitors tariquidar and PSC-833 plus the ABCC inhibitor MK571. The accumulation of both drugs was analysed in hCMEC/D3 cells after 30 minutes at 37 °C. Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with *** for $p<0.001$

To further characterise the unknown TPM and PHT transport processes, the effect of temperature was analysed as shown in Figure 3.5 A+B. An opposite effect as with chemical inhibitors was observed when temperature was lowered from 37 °C to 4 °C, with reduced TPM accumulation and elevated PHT accumulation. These results indicate that more than one transport process might be involved in TPM and PHT accumulation and both, uptake and efflux, are important in determining the intracellular drug concentrations. To gain more information about

the underlying transport kinetics, the accumulation of both drugs was determined at different time points, 0.25, 0.5, 1, 2, 5, 10, 20, and 30 minutes (Figure 3.5 C+D). While TPM exhibited typical transporter kinetics starting with a linear phase of uptake and followed by a saturation process (Figure 3.5 C), PHT uptake was much quicker and steady-state was reached after only 0.5 minutes (Figure 3.5 D).

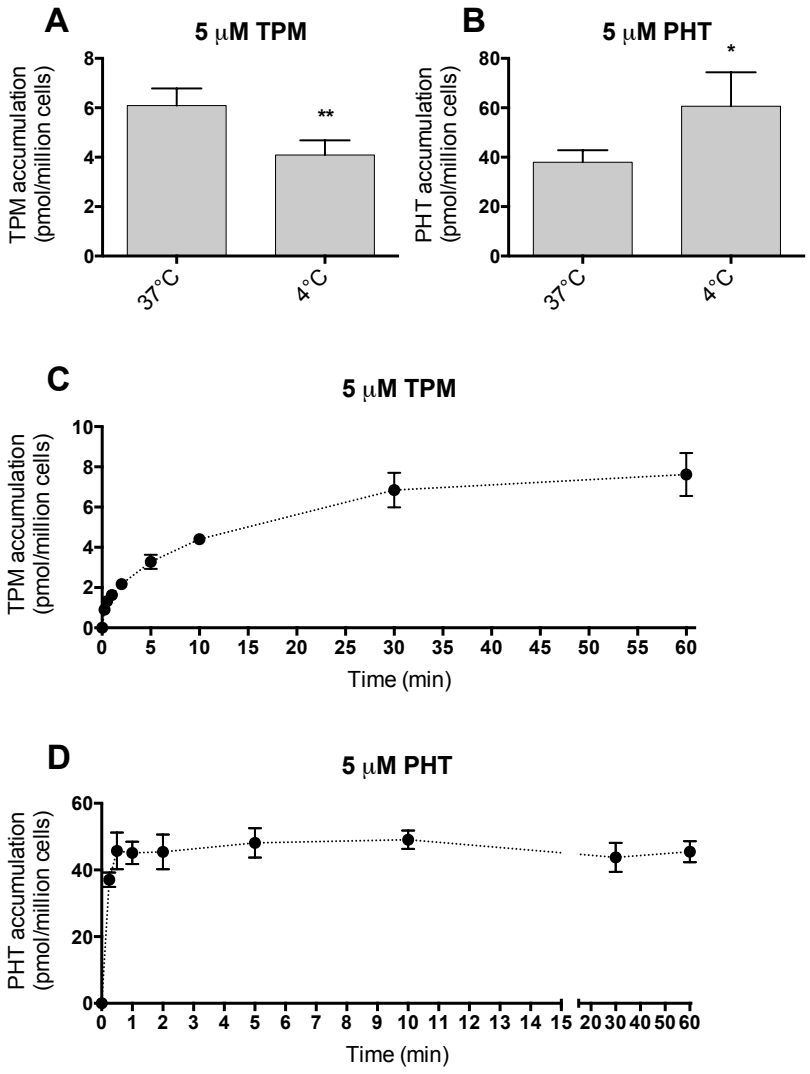


Figure 3.5: Effect of temperature and time on topiramate and phenytoin accumulation

Accumulation of A) topiramate (TPM) and B) phenytoin (PHT) in the hCMEC/D3 cell line after 30 minutes at 37 °C and 4 °C, respectively. Data are expressed as means ± standard deviation (n = 4 independent experiments in triplicate). Significant results are indicated with * for $p<0.05$ and ** for $p<0.01$. Time course of C) topiramate and D) phenytoin accumulation in the hCMEC/D3 cell line at 37 °C. Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate).

3.3.4. Gene expression of *ABCC* transporters in the hCMEC/D3 cell line

Based on the results from both screening approaches and clinical aspects, TPM was considered the most promising and more important AED candidate for further investigation. First, the transporter involved in TPM efflux appears to be a member of the ABCC transporter subfamily while the transporter involved in PHT uptake is less conclusive, possibly a member from the SLCO (OATP) superfamily. TPM efflux across the BBB is important to study further as it is a potential mechanism or contributing factor for TPM treatment resistance. Second, while PHT used to be a very important drug for the management of epilepsy, it is no longer recommended as first-line or second-line treatment for any epilepsy syndrome or seizure type in the UK (except as second-line treatment for *status epilepticus*), and hence is clinically less important than TPM (National Institute for Health and Clinical Excellence 2012).

Assuming that an ABCC transporter is involved in TPM efflux in the hCMEC/D3 cell line, a functional siRNA screening approach was considered a promising strategy to determine the exact transporter(s) for further characterisation. To reduce the number of transporters to be screened, the gene expression of all twelve known *ABCC* transporter genes (excluding one pseudogene) was determined in the hCMEC/D3 cell line using highly specific and sensitive TaqMan® gene expression assays with 100 ng cDNA per reaction. *ABCC* gene expression was normalised to *GAPDH* and plotted as reciprocal ΔC_t (Figure 3.6). A lower $1/\Delta C_t$ indicates lower gene expression. *ABCC2* showed borderline expression with a $1/\Delta C_t$ of 0.06 (*ABCC2* C_t was 39). No gene expression could be detected for *ABCC7*, *ABCC8*, *ABCC9*, *ABCC11*, and *ABCC12*.

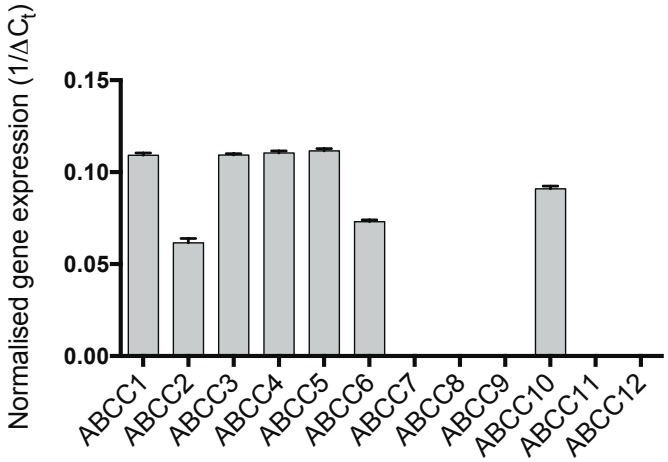


Figure 3.6: Gene expression of *ABCC* transporters in the hCMEC/D3 cell line normalised to *GAPDH*
Gene expression of all known human *ABCC* transporters (excluding pseudogene *ABCC13*) in the hCMEC/D3 cell line normalised to human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The normalised gene expression is given as the reciprocal delta of both threshold cycles ($1/\Delta C_t$). Empty fields indicate non-detectable *ABCC* gene expression. Data are expressed as means ± standard deviation and were derived from 1 experiment analysed in quadruplicate

3.3.5. siRNA assay optimisation

siRNA pools containing four targeting sequences were ordered for each expressed *ABCC* gene based on the results obtained in 3.3.4. Before screening, the assay had to be optimised for every gene to obtain the best possible knockdown efficiency. Knockdown efficiency is defined as the relative gene expression in cells transfected with targeting siRNAs as compared to cells transfected with non-targeting, scrambled siRNAs and calculated by subtracting both ΔC_t values ($\Delta\Delta C_t$). A knockdown efficiency with a remaining relative gene expression of ≤ 25 % was considered optimal. Initially, the siRNA delivery into hCMEC/D3 cells was optimised using *ABCC4* targeting siRNAs. siRNA delivery is particularly dependent on cell density as well as the amount of transfection reagent. All siRNA screening experiments were carried out on 6-well plates. Seeding of two different cell quantities, 0.1 million and 0.25 million, respectively, was analysed with regards to knockdown efficiency using 10 nM, 25 nM, and 50 nM of *ABCC4* targeting and control siRNAs, respectively (Figure 3.7 A). While the siRNA concentrations had no

effect on knockdown efficiency for this particular gene, a lower cell quantity of only 0.1 million cells per well resulted in a considerably higher knockdown efficiency. For one condition (0.25 million cells, 10 nM siRNA) the RNA pellet was lost during extraction and the relative gene expression could not be determined. This does, however, not affect the conclusions drawn.

According to the manufacturer, a final transfection reagent (DharmaFECT1) concentration between 0.05-0.5 % (v/v) is recommended for 6-well plates, depending on the cell line. For the initial experiment shown in Figure 3.7 A, 0.2 % (v/v) transfection reagent was chosen as a starting concentration. Using 0.1 million cells for plating and varying DharmaFECT1 concentrations between 0.05-0.4 % (v/v), the initially chosen concentration of 0.2 % (v/v) turned out to be optimal (Figure 3.7 B). After optimising the siRNA delivery into hCMEC/D3 cells, the ideal siRNA concentrations were determined for each particular *GoI*. Three siRNA concentrations were chosen to be tested, 10 nM, 25 nM, and 50 nM, and the knockdown efficiencies analysed (Figure 3.7 C). 25 nM siRNAs turned out to be optimal for the knockdown of all *ABCC* genes tested.

For *ABCC2*, no accurate comparative gene expression could be determined as the C_t values highly fluctuated around approximately 40 with both positive and negative $\Delta\Delta C_t$ values. According to the manufacturer, a C_t above 38 is considered as a "not detectable amplification signal" (Applied Biosystems by Life Technologies 2010). In addition, assuming very borderline *ABCC2* expression, such a low copy number will require a much larger sample size to improve the reliability of the results (Applied Biosystems by Life Technologies 2011). Therefore, the potential role of ABCC2 (MRP2) for TPM efflux can be considered negligible and results from chapter 2 of this thesis confirm that ABCC2 is not involved in TPM uptake into ABCC2 overexpressing plasma membrane vesicles (see chapter 2 section 2.3.2). The missing information for *ABCC1* knockdown efficiency using 50 nM siRNAs is due to a lost RNA pellet during RNA extraction. However, 25 nM siRNAs already yielded a high knockdown efficiency with only 12 % relative remaining gene expression and the experiment was therefore not repeated.

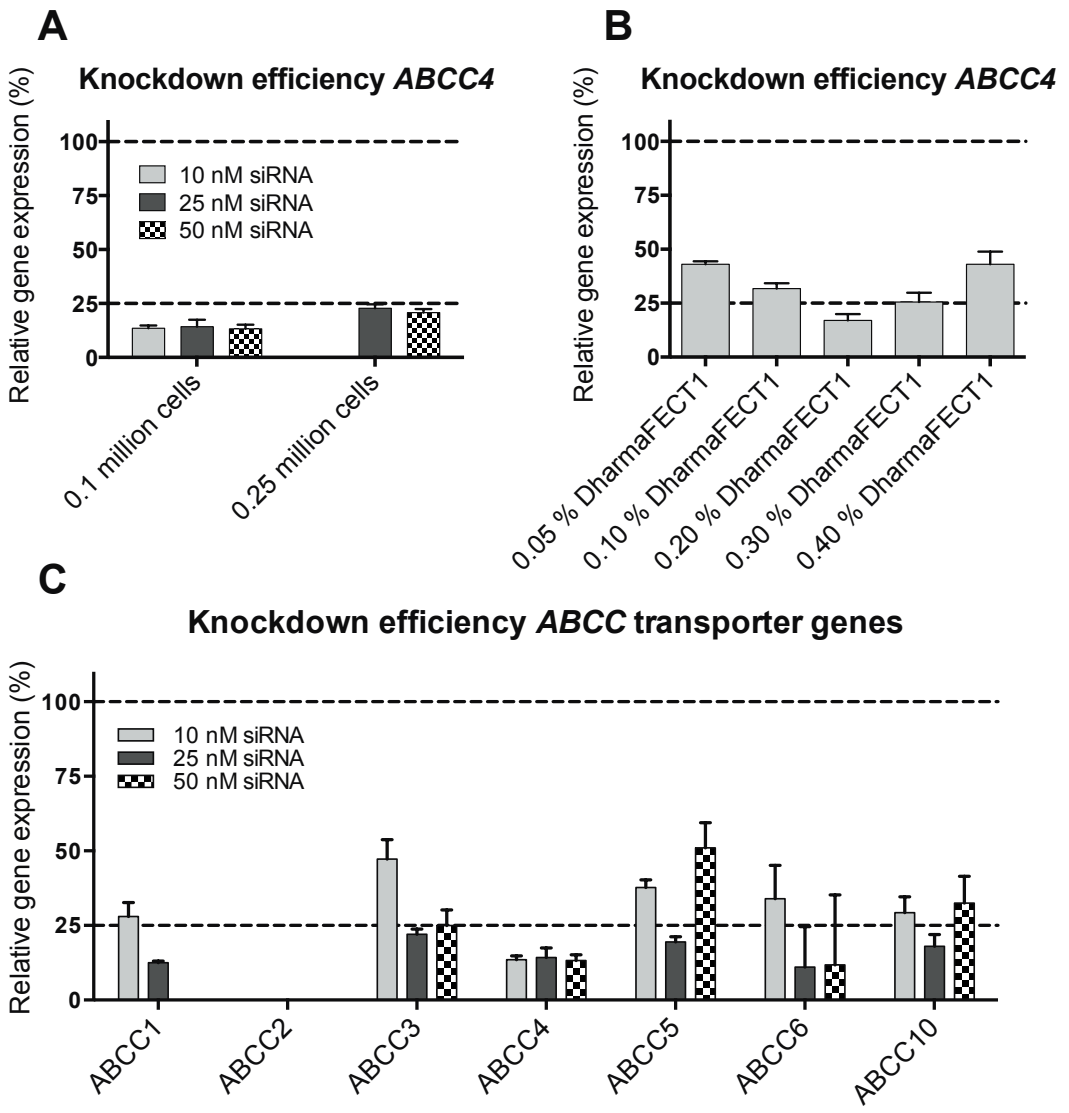


Figure 3.7: Optimisation of siRNA screening conditions for the hCMEC/D3 cell line

A) Knockdown efficiency utilising three concentrations of *ABCC4* targeting siRNAs (10 nM, 25 nM, 50 nM, respectively) and two different cell quantities (0.1 million and 0.25 million, respectively); B) Knockdown efficiency of *ABCC4* targeting siRNAs (10 nM) utilising varying transfection reagent concentrations. 0.1 million cells were plated as assessed in A; C) Knockdown efficiency of various *ABCC* genes under optimised delivery conditions (0.1 million plated cells, 0.2 % transfection reagent) using three siRNA concentrations, 10 nM, 25 nM, and 50 nM, respectively. Knockdown efficiency is plotted as the remaining relative gene expression in % as compared to negative control siRNA transfected cells. Analysis of *ABCC2* failed due to the borderline gene expression. Data are expressed as means ± standard deviation and were derived from 1 experiment analysed in quadruplicate

3.3.6. siRNA screening

TPM accumulation was assessed in the hCMEC/D3 cell line after 30 minutes at 37 °C utilising a siRNA screening approach under optimised conditions (3.3.5). No enhanced TPM accumulation could be observed after knockdown of any of the expressed *ABCC* transporter genes as compared to control (Figure 3.8 A). Gabapentin (GBP) has recently been demonstrated to be a substrate of the L- alpha amino acid transporter 1 (SLC7A5, LAT1) utilising a similar screening approach and was included as a functional positive control (Dickens *et al.* 2013a). Knockdown of *SLC7A5* resulted in a significantly reduced accumulation of GBP and confirmed the functionality of the assay (Figure 3.8 B).

The average knockdown efficiency was determined by real-time PCR and good efficiencies obtained for most genes tested (Figure 3.8 C). The average knockdown efficiency for *ABCC10* was 32 % remaining relative gene expression and therefore slightly higher than the threshold of 25 %. However, an average of 32 % was still considered acceptable as 25 % is a rather strict threshold and others have commonly used 30 % (Krueger *et al.* 2007). It is unlikely that 7 % or 2 % (25 % or 30 % threshold) would change the interpretation of the results as no tendency for an increased TPM accumulation could be observed in the functional assay. *ABCC5* knockdown, in contrast, was clearly insufficient with a high intra-experimental fluctuation (Figure 3.8 C). In addition, the functional assay resulted in a significantly decreased accumulation of TPM (Figure 3.8 A), possibly a siRNA off-target effect. It is unlikely that an increase in siRNA concentration will improve the knockdown efficiency without increasing the possible off-target effect. Therefore, a different pool of four *ABCC5* targeting siRNAs and corresponding negative control was ordered from a different manufacturer (Qiagen). Transfection of hCMEC/D3 cells using the same optimised conditions as before resulted in no measurable knockdown using 10 nM or 25 nM siRNAs. In conclusion, *ABCC5* gene expression seems to be difficult to target and further siRNA approaches are thus not considered promising. However, in a previous study using stably *ABCC5*-transfected cells it was demonstrated that TPM is not a substrate for this particular transporter

(Luna-Tortos *et al.* 2009). As before with the optimisation experiments, *ABCC2* gene expression is, at best, borderline and no accurate comparative gene expression could be determined (see for 3.3.5 discussion). Nevertheless, the functional screening with *ABCC2* targeting siRNAs was included as an additional negative control.

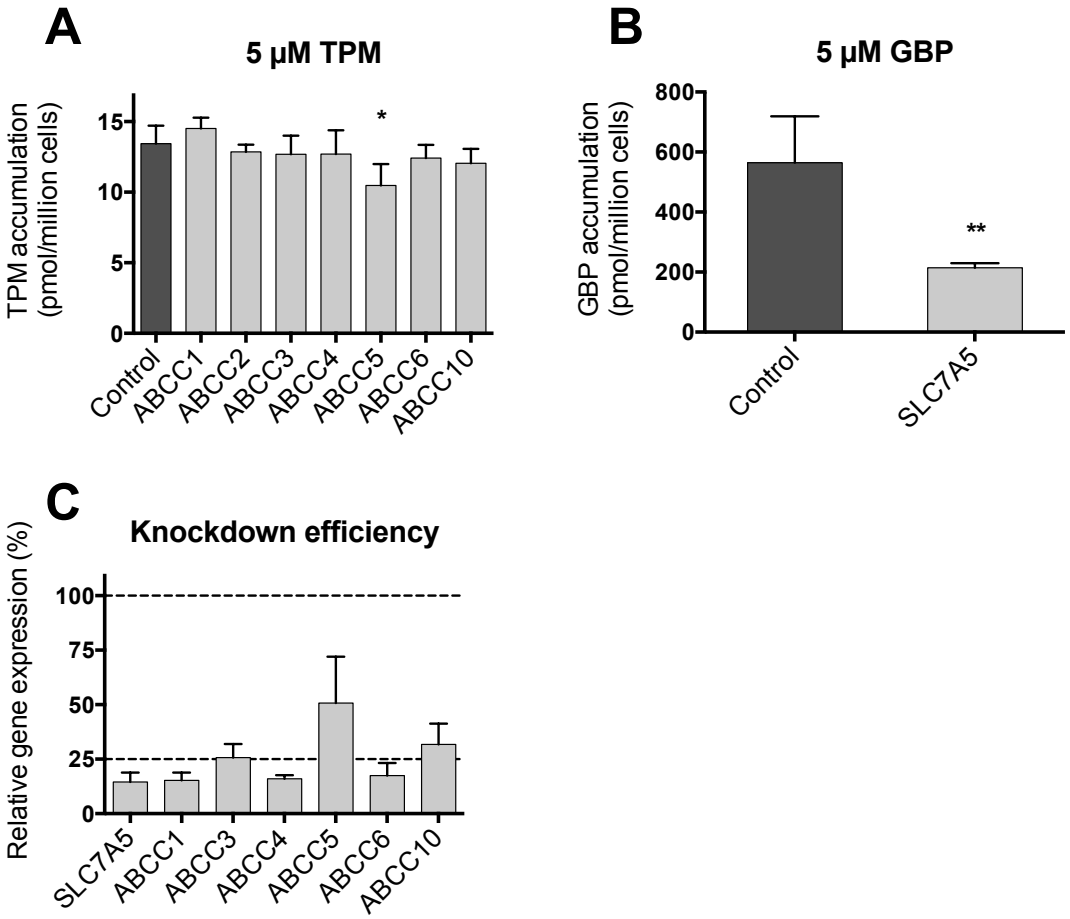


Figure 3.8: Functional siRNA screening of ABCC transporters in the hCMEC/D3 cell line

A) Accumulation of topiramate (TPM) in hCMEC/D3 cells after 30 minutes at 37 °C utilising a siRNA screening approach. Cells were transfected either with negative control siRNAs or *ABCC* transporter-targeting siRNAs; B) Functional positive control assay. The accumulation of gabapentin (GBP) in hCMEC/D3 cells transfected with control siRNA or *SLC7A5*-targeting siRNA was assessed after 30 minutes at 37 °C; C) Relative remaining gene expression as compared to negative control in %. The analysis of *ABCC2* failed due to the borderline gene expression. Data are expressed as means ± standard deviation (n= 4 independent experiments each in triplicate (A+B) or quadruplicate (C)). Significant results are indicated with * for $p<0.05$ and ** for $p<0.01$

3.4. Discussion

A screening approach by means of chemical transporter inhibitors and siRNAs was applied to identify unknown transport processes for already licensed AEDs. Although a variety of known chemical transporter inhibitors were chosen, the overall effect on drug accumulation was surprisingly modest and no effect could be observed for CBZ and VPA. Interestingly, VPA uptake into hCMEC/D3 cells was very low and detection close to background levels. *In vivo*, however, VPA is known to cross the BBB and is a very effective drug, particularly for the treatment of generalised seizures (see chapter 1 section 1.3.3.5). These results demonstrate that, as with every *in vitro* model, differences to the *in vivo* situation exist. However, *in vitro* models are useful tools for the initial identification of potential clinical effects (Giacomini *et al.* 2010), the main target for this research chapter.

Chemical inhibitors can never be considered absolutely specific and most of those tested have been demonstrated before to affect multiple drug transporter classes (3.3.2 and 3.3.3). While this is an issue for the identification of a particular transporter, it increases the probability of obtaining positive results. The overall accumulation profile may then help to identify the potentially involved group of transporters before more elaborate and expensive methods are considered. However, it appears that none of these groups is heavily involved in the accumulation of the six AEDs tested *in vitro* by using the hCMEC/D3 cell line as a BBB model. An exception was PHT with a reduced accumulation in the presence of several inhibitors tested. Also, TPM exhibited an interesting increase in accumulation in the presence of the prototypical ABCC transporter inhibitors MK571 and montelukast. Based on the inhibitor profile and clinical aspects (discussed in section 3.3.3), TPM was considered the most promising candidate AED to be further investigated as a potential substrate for one or more ABCC transporter(s).

RNAi is a useful method to screen the transporter-specific effect on drug accumulation for several drug transporters in parallel by means of siRNA

transfection (Dickens *et al.* 2013a). This method was applied on all expressed *ABCC* transporter genes but no increased TPM accumulation could be observed. Several reasons might have led to these negative results. First, the transporter affected by MK571 and montelukast could be from a different ABC subfamily. Also, an uptake transporter from the SLC superfamily might be involved and an allosteric rather than an inhibitory effect responsible for the increased TPM accumulation in the presence of MK571 and montelukast, respectively (Kindla *et al.* 2011). Second, although siRNA transfections substantially lowered the expression of the targeted transporter gene, the remaining expressed protein might still have enough capacity in the functional assay. In addition, functional redundancy could mask increased accumulation if the expression of only one transporter is impaired. For example, ABCC3 is well known to compensate for the impaired conjugated-bilirubin efflux from hepatocytes in patients with Dubin-Johnson syndrome (see chapter 1 section 1.2.2.2).

In conclusion, an interesting enhanced accumulation of TPM in the presence of MK571 and montelukast was observed in the hCMEC/D3 cell line as an *in vitro* model of the BBB. A subsequent siRNA screening, however, failed to identify the exact transporter(s) involved in TPM efflux and it remains unclear if it/they belong(s) to the ABCC subfamily as hypothesised from the inhibitor screening. Assays with transient transfections of every expressed *ABCC* transporter may be a strategy to overcome the limitations of RNAi. However, these assays will also give negative results if the involved transporter(s) is/are from a different ABC subfamily or even from the SLC superfamily. A large-scale siRNA library screening approach targeting all expressed ABC transporters could be an alternative but with the same limitations as discussed above.

3.5. References

Allen, J. D.*, et al.* (2002) Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Molecular cancer therapeutics* **1**, 417-425

Applied Biosystems by Life Technologies (2011) Real-time PCR: Understanding Ct http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_053906.pdf

Applied Biosystems by Life Technologies (2010) White Paper TaqMan(R) Assays QPCR Guarantee Program http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_088754.pdf

Badagnani, I.*, et al.* (2006) Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J Pharmacol Exp Ther* **318**, 521-529

Bailey, D. G.*, et al.* (2007) Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice. *Clinical pharmacology and therapeutics* **81**, 495-502

Burckhardt, G., and Burckhardt, B. C. (2011) In vitro and in vivo evidence of the importance of organic anion transporters (OATs) in drug therapy. *Handb.Exp.Pharmacol.*, 29-104

Chen, Z. S.*, et al.* (2003) Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. *Cancer Res* **63**, 4048-4054

Davies, A.*, et al.* (2009) Nilotinib concentration in cell lines and primary CD34(+) chronic myeloid leukemia cells is not mediated by active uptake or efflux by major drug transporters. *Leukemia* **23**, 1999-2006

Dickens, D.*, et al.* (2012) Lamotrigine is a substrate for OCT1 in brain endothelial cells. *Biochemical pharmacology* **83**, 805-814

Dickens, D.*, et al.* (2013a) Transport of gabapentin by LAT1 (SLC7A5). *Biochemical pharmacology* **85**, 1672-1683

Dickens, D.*, et al.* (2013b) A multi-system approach assessing the interaction of anticonvulsants with P-gp. *PloS one* **8**, e64854

Dresser, G. K.*, et al.* (2002) Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clin.Pharmacol.Ther.* **71**, 11-20

Giacomini, K. M.*, et al.* (2010) Membrane transporters in drug development. *Nat.Rev.Drug Discov.* **9**, 215-236

Janssen Pharmaceuticals Inc. (2012) Topamax Prescribing Information

Kenakin, T. P. (2012) Pharmacology in Drug Discovery: Understanding Drug Response, *Elsevier, Inc.*

Keppler, D. (2011) Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handbook of experimental pharmacology*, 299-323

Khamdang, S.*, et al.* (2002) Interactions of human organic anion transporters and human organic cation transporters with nonsteroidal anti-inflammatory drugs. *J Pharmacol Exp Ther* **303**, 534-539

Kindla, J.*, et al.* (2011) Influence of non-steroidal anti-inflammatory drugs on organic anion transporting polypeptide (OATP) 1B1- and OATP1B3-mediated drug transport. *Drug metabolism and disposition: the biological fate of chemicals* **39**, 1047-1053

Krueger, U.*, et al.* (2007) Insights into effective RNAi gained from large-scale siRNA validation screening. *Oligonucleotides* **17**, 237-250

Lagas, J. S.*, et al.* (2009) Transport of diclofenac by breast cancer resistance protein (ABCG2) and stimulation of multidrug resistance protein 2 (ABCC2)-mediated drug transport by diclofenac and benzbromarone. *Drug metabolism and disposition: the biological fate of chemicals* **37**, 129-136

Letschert, K.*, et al.* (2006) Molecular characterization and inhibition of amanitin uptake into human hepatocytes. *Toxicological sciences : an official journal of the Society of Toxicology* **91**, 140-149

Letschert, K.*, et al.* (2005) Vectorial transport of the peptide CCK-8 by double-transfected MDCKII cells stably expressing the organic anion transporter OATP1B3 (OATP8) and the export pump ABCC2. *J Pharmacol Exp Ther* **313**, 549-556

Luna-Tortos, C.*, et al.* (2009) The antiepileptic drug topiramate is a substrate for human P-glycoprotein but not multidrug resistance proteins. *Pharm.Res.* **26**, 2464-2470

Mandery, K.*, et al.* (2010) Influence of the flavonoids apigenin, kaempferol, and quercetin on the function of organic anion transporting polypeptides 1A2 and 2B1. *Biochemical pharmacology* **80**, 1746-1753

Maryanoff, B. E.*, et al.* (1998) Structure-activity studies on anticonvulsant sugar sulfamates related to topiramate. Enhanced potency with cyclic sulfate derivatives. *J Med Chem* **41**, 1315-1343

Matsson, P.*, et al.* (2007) A global drug inhibition pattern for the human ATP-binding cassette transporter breast cancer resistance protein (ABCG2). *J.Pharmacol.Exp.Ther.* **323**, 19-30

Matsson, P.*, et al.* (2009) Identification of novel specific and general inhibitors of the three major human ATP-binding cassette transporters P-gp, BCRP and MRP2 among registered drugs. *Pharm.Res.* **26**, 1816-1831

National Institute for Health and Clinical Excellence (2012) The epilepsies: the diagnosis and management of the epilepsies in adults and children in primary and secondary care (NICE clinical guideline 137)

Nies, A. T.*, et al.* (2011) Organic Cation Transporters (OCTs, MATEs), In Vitro and In Vivo Evidence for the Importance in Drug Therapy. *Handb.Exp.Pharmacol.* **201**, 105-167

Ohashi, R.*, et al.* (1999) Na(+)-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. *J Pharmacol Exp Ther* **291**, 778-784

Reid, G.*, et al.* (2003) The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc.Natl.Acad.Sci.U.S.A* **100**, 9244-9249

Roth, M., Obaidat, A., and Hagenbuch, B. (2011) OATPs, OATs and OCTs: The organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br.J.Pharmacol.*

Roy, U.*, et al.* (2009) Montelukast is a potent and durable inhibitor of multidrug resistance protein 2-mediated efflux of taxol and saquinavir. *Biol.Pharm.Bull.* **32**, 2002-2009

Shirasaka, Y.*, et al.* (2009) Concentration-dependent effect of naringin on intestinal absorption of beta(1)-adrenoceptor antagonist talinolol mediated by p-glycoprotein and organic anion transporting polypeptide (Oatp). *Pharmaceutical research* **26**, 560-567

Uwai, Y.*, et al.* (2007) Interaction and transport characteristics of mycophenolic acid and its glucuronide via human organic anion transporters hOAT1 and hOAT3. *Biochemical pharmacology* **74**, 161-168

van de Waterbeemd, H.*, et al.* (1998) Estimation of blood-brain barrier crossing of drugs using molecular size and shape, and H-bonding descriptors. *Journal of drug targeting* **6**, 151-165

Weksler, B. B.*, et al.* (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 1872-1874

Wolff, N. A.*, et al.* (2007) Mycophenolic acid (MPA) and its glucuronide metabolites interact with transport systems responsible for excretion of organic anions in the basolateral membrane of the human kidney. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **22**, 2497-2503

Wu, L. X.*, et al.* (2012) Effects of natural products on the function of human organic anion transporting polypeptide 1B1. *Xenobiotica* **42**, 339-348

Yabuuchi, H.*, et al.* (1999) Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations. *J Pharmacol Exp Ther* **289**, 768-773

Yunger, L. M., and Cramer, R. D., 3rd (1981) Measurement of correlation of partition coefficients of polar amino acids. *Molecular pharmacology* **20**, 602-608

Zhang, S., and Morris, M. E. (2003) Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *J Pharmacol Exp Ther* **304**, 1258-1267

Zhang, S., Yang, X., and Morris, M. E. (2004) Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Molecular pharmacology* **65**, 1208-1216

Chapter 4

Drug-drug interactions with lamotrigine mediated by SLC22A1 (OCT1)

Contents

4.1. Introduction

LTG transport by SLC22A1 (OCT1) has recently been demonstrated *in vitro* utilising an *SLC22A1*-transfected chronic myeloid leukemia cell line (KCL22) and the hCMEC/D3 cell line as a BBB model (Dickens *et al.* 2012). A large number of drugs, particularly organic cations or drugs that display a positive net charge at physiological pH, have been demonstrated to act as inhibitors of SLC22A1 (see chapter 1 section 1.2.1.3). Therefore, the potential for SLC22A1-mediated drug-drug interactions with LTG is high. Two clinically relevant interactions were noted from the literature and analysed in this research chapter.

In a study with healthy volunteers, LTG serum levels were significantly changed in subjects co-treated with lopinavir/ritonavir as compared to treatment with LTG only (van der Lee *et al.* 2006). The area under the curve (AUC) was reduced by 50 % and twice the LTG dose was required to achieve serum levels comparable to LTG monotherapy (van der Lee *et al.* 2006). Lopinavir/ritonavir is an antiretroviral protease inhibitor combination indicated for the treatment of HIV-1. In the US and European Economic Area it is marketed by AbbVie under the brand name Kaletra® (AbbVie Inc. 2013). While the antiretroviral activity of Kaletra® is mediated by lopinavir, ritonavir is co-formulated to boost the plasma levels of lopinavir by means of CYP3A enzyme inhibition (AbbVie Inc. 2013).

So far, no data for a potential lopinavir and SLC22A1 interaction have been published, but two *in vitro* studies reported an inhibitory effect of ritonavir on the uptake of two SLC22A1 model substrates, tetraethylammonium (TEA^+) and MPP^+ (Jung *et al.* 2008, Jung *et al.* 2012, Zhang *et al.* 2000). Individuals infected with HIV often experience seizure disorders with an incidence of 11 % (Birbeck *et al.* 2012). Interactions between AEDs and antiretroviral drugs are thus important to understand, particularly with LTG as a first-line treatment option for focal seizures and important alternative to VPA for generalised seizures (National Institute for Health and Clinical Excellence 2012). The decreased LTG serum levels in the presence of lopinavir/ritonavir have been linked to an enhanced LTG metabolism

with elevated 2N-glucuronide concentrations (AbbVie Inc. 2013, Burger *et al.* 2008, GlaxoSmithKline Inc. 2012, van der Lee *et al.* 2006). However, an impaired LTG uptake by SLC22A1 could be an additional factor leading to the observed alterations.

The atypical antipsychotic clozapine (CLP) (introduced in more detail in chapter 5) is indicated as a last-resort treatment option for otherwise refractory schizophrenia (Novartis Pharmaceuticals Corporation 2013). Despite this option, there is still a large proportion of patients that also fail to respond to CLP (Chakos *et al.* 2001, Kane *et al.* 1988, Lieberman *et al.* 1994). To improve treatment response, different CLP augmentation strategies have been reported, including the AED LTG. A meta-analysis from 2009 (Tiihonen *et al.* 2009) that analysed five clinical studies (Goff *et al.* 2007, Kremer *et al.* 2004, Tiihonen *et al.* 2003, Zoccali *et al.* 2007) found a significant superiority of LTG augmentation in comparison to placebo. Recently, another meta-analysis from the same five studies reported contradictory findings (Sommer *et al.* 2012). By analysing the effect size of each study, one (Zoccali *et al.* 2007) was identified as an outlier of more than two SDs from the mean weighted effect size and excluded from further analysis (Sommer *et al.* 2012). The remaining studies did not show superiority of LTG over placebo augmentation. However, if the largest and longest randomised controlled trial from the entire group is excluded as a statistical outlier, this could bias the results. Another meta-analysis on a subset of these studies (Goff *et al.* 2007, Tiihonen *et al.* 2003) did not find superiority of LTG augmentation as compared to placebo (Porcelli *et al.* 2012).

In conclusion, LTG may be beneficial as an augmentation strategy to overcome CLP resistance, at least for a subset of patients, but more clinical studies are needed. Assuming there is a positive effect, the underlying molecular reason would be important to understand. LTG is a substrate of SLC22A1 and another atypical antipsychotic drug, quetiapine, has been characterised as a potent inhibitor of this particular transport (Dickens *et al.* 2012). Thus, an interaction between LTG and CLP on the drug transporter level is hypothesised to contribute to an altered CLP treatment efficacy. Notably, a clinical interaction between quetiapine and LTG has also been reported (Andersson *et al.* 2011, Castberg *et al.* 2007).

4.2. Materials and methods

4.2.1. Materials

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich Company Ltd., Gillingham, UK, or listed in the corresponding section of chapter 3 (3.2.1). Tritium-labelled CLP ([^{3}H]-CLP, 1 mCi/ml, specific activity 80.0 Ci/mmol) and carbon-14-labelled TEA^{+} ([^{14}C]-TEA, 0.1 mCi/ml, specific activity 55.0 mCi/mmol) were obtained from American Radiolabeled Chemicals Inc., St. Louis, MO, USA. Lopinavir, ritonavir, lamivudine, and abacavir came from Insight Biotechnology Ltd., Wembley, UK, L-glutamine (200 mM) from Life Technologies Ltd., Paisley, UK, and BD Matrigel^{TM} Basement Membrane Matrix from BD Biosciences, Oxford, UK.

4.2.2. Cell lines and culture conditions

4.2.2.1. hCMEC/D3 cell line

Please refer to chapter 3 section 3.2.2.1.

4.2.2.2. KCL22 cell lines

The human chronic myeloid leukaemia cell line KCL22 was stably transfected with an empty vector (control) or a vector encoding for SLC22A1 (OCT1) by means of electroporation (Giannoudis *et al.* 2008). Both non-adherent cell lines were a kind gift from Dr Athina Giannoudis (Department of Haematology, University of Liverpool, UK) and cultured in RPMI-1640 medium supplemented with 10 % FBS (v/v) and 1 % penicillin-streptomycin (v/v) at 37 °C and 5 % CO_2.

4.2.2.3. Primary human hepatocytes

Primary human hepatocytes were derived from patients that underwent surgery for resectable primary hepatocellular carcinoma or colorectal liver metastases. All patients gave their written informed consent to the use of their resected tissue for experimental purposes and the Liverpool Central Research Ethics Committee gave

ethical approval. Hepatocytes were extracted with kind help of Dr Rowena Sison-Young and James Heslop.

Excess healthy liver parenchyma was resected as part of the regular procedure and immediately stored in ice-cold HEPES buffer (10 mM HEPES, 136 mM NaCl, 5 mM KCl, 0.5 % glucose, pH 7.6) on ice. Within 30 minutes of removal from the patient, the extraction process for primary human hepatocytes was initiated by perfusion of accessible circulatory vessels to wash the tissue. Perfusion was carried out at a flow rate of 50 ml/min using 1-2 l warm (37 °C) HEPES buffer. After washing, the perfusion continued for up to 20 minutes with the addition of 700 µM $CaCl_2$ and 0.5 mg/ml collagenase to digest the connective tissue. The digested tissue was then carefully disrupted using forceps and blunt-ended scissors and the cells disassociated with ice-cold Williams' medium E without L-glutamine. The cells were poured through a nylon mesh and centrifuged at 80 g for 5 minutes at 4 °C (Megafuge 11R centrifuge with T41 swing-out rotor, Thermo Fisher Scientific, Leicestershire, UK). The resulting cell pellet was carefully resuspended in ice-cold William´s medium E without L-glutamine by gently rocking back and forth. Finally, the cells were centrifuged as before and resuspended in full William´s medium E (supplemented with 1 % penicillin-streptomycin (v/v), 2 mM L-glutamine, 1 % of 100x insulin-transferrin-selenium (ITS) liquid media supplement (v/v), 100 nM dexamethasone). Cell viability and concentration was determined using a Countess® Automated Cell Counter (Life Technologies Ltd., Paisley, UK) after staining with trypan blue. A cell viability of ≥ 85 % was required for further experimentation.

Initially, 500,000 cells were seeded into each well of ready to use collagen-coated (collagen I from rat tail) 24-well plates (Life Technologies Ltd., Paisley, UK) and incubated at 37 °C and 5 % CO_2 for 3 hours. The medium was removed, cells washed once with Willliam´s medium E without L-glutamine, and hepatocytes finally incubated for 12 hours at 37 °C and 5 % CO_2 in full William´s medium E (supplemented with 1 % penicillin-streptomycin (v/v), 2 mM L-glutamine, 1 % of 100x ITS liquid media supplement (v/v), 100 nM dexamethasone).

4.2.2.4. Primary rat hepatocytes

Primary rat hepatocytes (kindly provided by Dr Sophie Regan) were derived from adult male Wistar rats (175 – 300 g) obtained from Charles River Laboratories (Margate, Kent, UK). All experiments involving live animals were carried out according to criteria outlined in a license that was granted under the Animals (Scientific Procedures) Act 1986 and approved by the Animal Ethics Committee of the University of Liverpool.

For hepatocyte extraction, animals were anaesthetised with sodium pentobarbital (1 µl/g) and the liver perfused with warm (37 °C) HEPES buffer (HBSS without Ca^{+} supplemented with 5.8 mM HEPES and 4.5 mM $NaHCO_3$) at a flow rate of 40 ml/min for 9 minutes. After this initial washing step, perfusion was continued as before but in the presence of 0.05 % collagenase (w/v), 0.0068 % trypsin inhibitor (w/v), and 5 mM $CaCl_2$ until sufficiently digested. Subsequently, the liver was removed from the animal and rinsed in HEPES buffer containing 0.1 mg/ml DNAse I (w/v). The cells were disassociated from connective tissue by careful combing with sharp forceps and poured through a nylon mesh before they were centrifuged at 50 g for 2 minutes at 4 °C (Heraeus™ Megafuge™ 11R centrifuge with T41 rotor, Thermo Fisher Scientific, Leicestershire, UK). The resulting cell pellet was washed twice with ice-cold HEPES buffer containing 0.1 mg/ml DNAse I. Cells were centrifuged between and after the washing steps as described before. Finally, the washed cell pellet was resuspended in full William´s medium E (supplemented with 1 % penicillin-streptomycin (v/v), 2 mM L-glutamine, 1.25 % of 100x ITS liquid media supplement (v/v), 25 nM dexamethasone, and 5 % FBS (v/v)). Cell viability and concentration was determined using a Countess® Automated Cell Counter (Life Technologies Ltd., Paisley, UK) after staining with trypan blue. A cell viability of ≥ 85 % was required for further experimentation..

Initially, 300,000 cells were seeded into each well of ready to use collagen-coated (collagen I from rat tail) 24-well plates (Life Technologies Ltd., Paisley, UK) and incubated at 37 °C and 5 % CO_2 for 3 hours. The medium was removed, cells washed once with Willliam´s medium E without L-glutamine, and

hepatocytes finally incubated for 12 hours at 37 °C and 5 % CO_2 in full William´s medium E (supplemented with 1 % penicillin-streptomycin (v/v), 2 mM L-glutamine, 1.25 % of 100x ITS liquid media supplement (v/v), 25 nM dexamethasone, and 5 % FBS (v/v)) including 0.5 mg/ml BD Matrigel™ Basement Membrane Matrix.

4.2.3. Drug accumulation assays

4.2.3.1. Non-adherent cells

As preparation for accumulation assays with the non-adherent KCL22 cell lines, each cell incubation was initially centrifuged at 250 g for 5 minutes at room temperature (Heraeus™ Megafuge™ 11R centrifuge with T41 swing-out rotor, Thermo Fisher Scientific, Leicestershire, UK). The culture medium was discarded and the cell pellets resuspended in pre-warmed (37 °C) HBSS followed by another centrifugation step. Washed cells were resuspended in pre-warmed (37 °C) transport buffer (HBSS supplemented with 25 mM HEPES, adjusted to pH 7.4) and the viability and concentration determined using a Countess® Automated Cell Counter (Life Technologies Ltd., Paisley, UK) after staining with trypan blue. A 250 µl cell suspension (adjusted to 8.0 million cells per ml) was used for each reaction and transferred into a 1.5 ml microcentrifuge tube (Starlab Ltd., Milton Keynes, UK) to give a final quantity of 2.0 million cells per reaction.

A double-concentrated master mix was prepared separately for each condition to be tested, consisting of transport buffer with 0.11 - 0.3 µCi/ml [^{14}C]-TEA^+ or 0.3 µCi/ml [^{3}H]-LTG, non-labelled corresponding “cold” drug to give 5.46 µM TEA^+ or 10 µM LTG, CLP as inhibitor from 1000x concentrated stock solutions, and 0.2 % bovine serum albumin (BSA) (w/v). The vehicle concentration did not exceed 1.1 % per reaction (ethanol and/or DMSO). The assay was initiated by adding 250 µl corresponding master mix to each tube containing 250 µl empty vector-transfected or *SLC22A1*-transfected KCL22 cells. Reactions were gently mixed, incubated for 30 minutes at 37 °C, and subsequently stored on ice for another 10 minutes. To remove excess radioactive drug, each reaction mix was washed three times with 1 ml ice-cold HBSS. Samples were centrifuged at 250 g for

5 minutes at 4 °C between and after the washing steps (Megafuge 11R centrifuge with T41 swing-out rotor, Thermo Fisher Scientific, Leicestershire, UK). Finally, the cells were lysed in 400 µl 10 % SDS and transferred into scintillation tubes containing 4 ml scintillation cocktail. Radioactivity was determined by scintillation counting (1500 Tri Carb LS Counter, Packard, now PerkinElmer, Seer Green, UK).

4.2.3.2. Adherent cells

Cell accumulation assays for adherent cells, namely hCMEC/D3, primary human hepatocytes, and primary rat hepatocytes were carried out as described in chapter 3 section 3.2.4. The master mix consisted of transport buffer (HBSS supplemented with 25 mM HEPES, adjusted to pH 7.4) with 0.3 µCi/ml [^{3}H]-LTG or 0.1 µCi/ml [^{3}H]-CLP, non-labelled corresponding "cold" drug to give 5 µM LTG or 1 µM CLP, corresponding CLP or LTG as inhibitor, and 0.1 % BSA (w/v). The vehicle concentration did not exceed 1.1 % per reaction (DMSO).

4.2.3.3. Poly-L-lysine-attached non-adherent cells

For time-course assays, quick washing of the cells is essential in order to obtain accurate data for shorter incubation times. To conduct time-course assays with the non-adherent KCL22 cell lines, cells were first attached to the surface of Nunc™ 6-well plates (Fisher Scientific UK Ltd., Loughborough, UK) by means of poly-L-lysine. A 1/20 dilution from a 0.01 % poly-L-lysine stock solution (w/v) was prepared and each well incubated with 1 ml for 10 minutes at room temperature. The solution was removed and plates left to air-dry. In the meantime, KCL22 cell cultures were prepared and initially centrifuged at 250 g for 5 minutes at room temperature (Megafuge 11R centrifuge with T41 swing-out rotor, Thermo Fisher Scientific, Leicestershire, UK). The culture medium was discarded and the cell pellets resuspended in pre-warmed (37 °C) HBSS followed by another centrifugation step. Washed cells were again resuspended in pre-warmed HBSS (37 °C) and the viability and concentration determined using a Countess® Automated Cell Counter (Life Technologies Ltd., Paisley, UK) after staining with trypan blue. The concentration was adjusted to 2 million cells per ml. 1 ml of the cell suspension was finally

transferred onto each well of air-dried poly-L-lysine-coated 6-well plates and incubated at 37 °C for 30 to 60 minutes to allow attachment of the cells.

The assay was initiated by replacing HBSS with master mix consisting of transport buffer (HBSS supplemented with 25 mM HEPES, adjusted to pH 7.4) with 0.05 µCi/ml [^{3}H]-CLP, non-labelled "cold" CLP to give a final concentration of 1 µM drug, and 0.1 % BSA (w/v). The vehicle concentration did not exceed 1.1 % per reaction (DMSO). Cells were incubated for the desired time at 37 °C and subsequently washed with 1 ml ice-cold HBSS. Washing was repeated three times and the cells finally lysed in 400 µl 10 % SDS. Each lysate was transferred into a scintillation tube containing 4 ml scintillation cocktail and radioactivity determined by scintillation counting (1500 Tri Carb LS Counter, Packard, now PerkinElmer, Seer Green, UK).

4.2.4. Gene expression

RNA extraction, RT-PCR, and real-time PCR were carried out as described in chapter 3 section 3.2.6. TaqMan® assay probe sequences and identification numbers are listed in the appendix. The human beta-actin (*ACTB*) gene expression assay was FAM™-labelled and therefore used in singleplex reactions. Each singleplex reaction consisted of 10 µl 2x concentrated TaqMan® master mix (1x final concentration), 1 µl *GoI* TaqMan® gene expression assay, 2 µl cDNA (50 ng/µl), and 7 µl molecular biology-grade water.

4.2.5. Statistical analysis

All data are presented as means ± SD. The inhibitor concentration that achieved half-maximum inhibition (IC_{50}) of substrate accumulation was determined by fitting a non-linear regression curve with variable slope to the data using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

4.3. Results

4.3.1. Interactions between lamotrigine and antiretrovirals mediated by SLC22A1 (OCT1)

The effect of lopinavir and ritonavir on SLC22A1 (OCT1)-mediated LTG uptake was analysed *in vitro* utilising *SLC22A1*-transfected and empty vector-transfected KCL22 cell lines. Two additional antiretroviral drugs, lamivudine and abacavir, were included as both have previously been shown to potently inhibit SLC22A1-mediated transport of MPP^+, an OCT model substrate (Jung *et al.* 2008, Minuesa *et al.* 2009). At a fixed concentration of 10 µM, lamivudine and abacavir had no effect on SLC22A1-mediated LTG uptake after 30 minutes at 37 °C (Figure 4.1 A). In the presence of 10 µM lopinavir and ritonavir, in contrast, a statistically significant decrease in LTG accumulation was observed as compared to control. Ritonavir was more potent than lopinavir with 56 % and 39 % reduction, respectively (Figure 4.1 A).

Clinically, lopinavir and ritonavir are used in combination with ritonavir as a pharmacokinetic booster for lopinavir plasma levels (Kaletra®). Following twice-daily dosing with 400/100 mg Kaletra®, the mean peak plasma concentrations of lopinavir and ritonavir reach about 13.5 µM and 0.8 µM at steady-state, respectively (AbbVie Inc. 2013). Thus, the ritonavir concentrations achieved in patients are much lower than 10 µM and an additional accumulation experiment was carried out in the presence of 13.5 µM lopinavir and 0.8 µM ritonavir (Figure 4.1 A). The same effect as with lopinavir alone (both resulted in a statistically significant 39 % reduction of LTG accumulation as compared to control) was observed, indicating that lopinavir is an inhibitor of SLC22A1-mediated LTG uptake at clinically relevant concentrations. To further characterise this effect, the SLC22A1-mediated uptake of LTG was determined in the presence of increasing lopinavir concentrations ranging from 0.1 – 100 µM (Figure 4.1 B). While the inhibitory effect seen before was confirmed, an IC_{50} could not be determined due to the high SD for some data points combined with a slowly decreasing accumulation

over the lopinavir concentration range used. In addition, the curve appears to reach a plateau and exhibit a biphasic dose response, although this observation has to be taken with care and may result from the high SD of some data points.

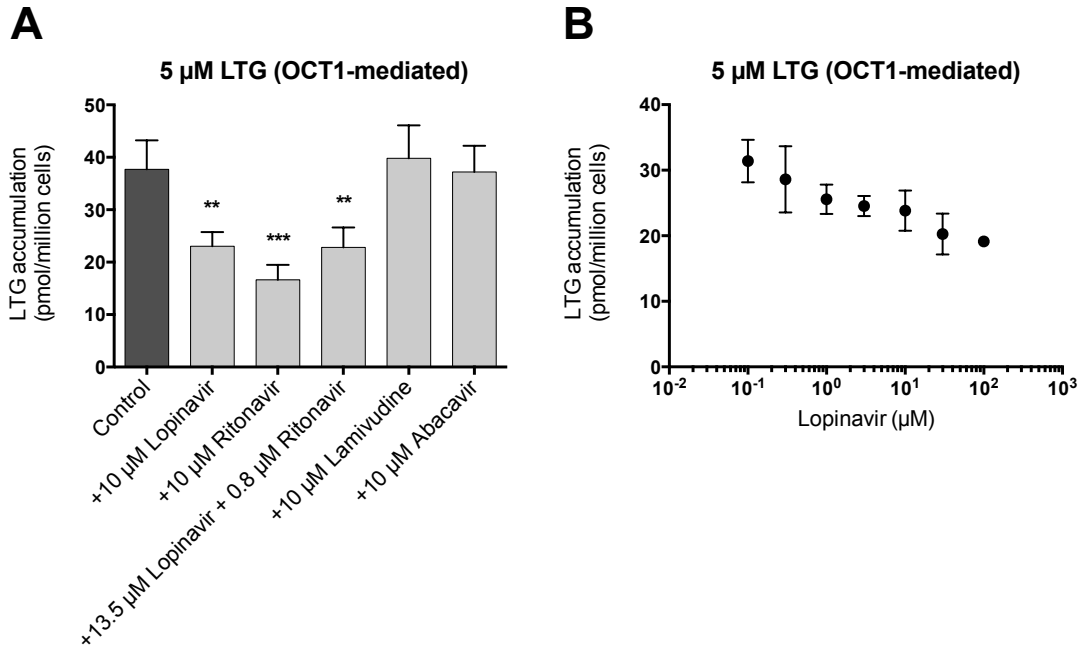


Figure 4.1: Interactions between lamotrigine and antiretrovirals mediated by SLC22A1 (OCT1)

A) The uptake of 5 µM lamotrigine (LTG) into KCL22 cells transfected with empty vector or *SLC22A1* was determined after 30 minutes at 37 °C in the presence of different antiretroviral drugs, namely lopinavir, ritonavir, lamivudine, and abacavir; B) The uptake of 5 µM lamotrigine into KCL22 cells transfected with empty vector or *SLC22A1* was determined after 30 minutes at 37 °C in the presence of increasing lopinavir concentrations (0.1 – 100 µM). SLC22A1 (OCT1)-mediated uptake was determined by subtracting the data of empty vector-transfected from *SLC22A1*-transfected KCL22 cells. Results are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with ** for $p<0.01$ and *** for $p<0.001$

4.3.2. Analysis of clozapine as a potential SLC22A1 (OCT1) substrate

To test the hypothesis that LTG enhances CLP effectiveness by means of an interaction with SLC22A1 (OCT1), CLP was initially investigated as a potential substrate for SLC22A1 in a time course assay utilising *SLC22A1*-transfected and

empty vector-transfected KCL22 cell lines. A CLP concentration as low as 1 µM (range from 0.3 – 2.4 µM) has been reported as average peak plasma concentration at steady-state following twice-daily doses of 100 mg (Novartis Pharmaceuticals Corporation 2013). This concentration was chosen for all assays presented in this research chapter. A time-course accumulation assay over 30 minutes at 37 °C with data obtained at 0.25, 0.5, 1, 2, 5, 10, 20, and 30 minutes did not show any significant difference between empty vector-transfected and *SLC22A1*-transfected KCL22 cell lines (Figure 4.2 A). A positive control utilising 5 µM LTG as a SLC22A1 substrate (30 minutes time point) confirmed the functionality of the assay (Figure 4.2 B). These results demonstrate that CLP is not a substrate for SLC22A1 *in vitro* using clinically relevant concentrations. Therefore, the hypothesis that CLP effectiveness is enhanced in the presence of LTG by means of an SLC22A1 interaction has to be rejected.

A transporter other than SLC22A1, however, may still affect CLP uptake into the brain. Therefore, the accumulation of CLP was assessed in the hCMEC/D3 cell line as an *in vitro* model of the BBB after 30 minutes at 37 °C in the presence of increasing LTG concentrations ranging from 1 – 1000 µM. An inhibitory effect was observed with LTG concentrations ≥ 100 µM, but not within therapeutic range. The manufacturer's product monograph specifies that LTG peak plasma levels range between 2 – 18 µM following single LTG doses of 50 – 400 mg (GlaxoSmithKline Inc. 2012). A peak plasma concentration up to 47 µM has been reported in a study that assessed individual therapeutic thresholds in epilepsy patients with doses up to 1,200 mg (Sondergaard Khinchi *et al.* 2008). This value represents an extreme and is indicated in Figure 4.2 C by a dotted line as maximum relevant LTG concentration.

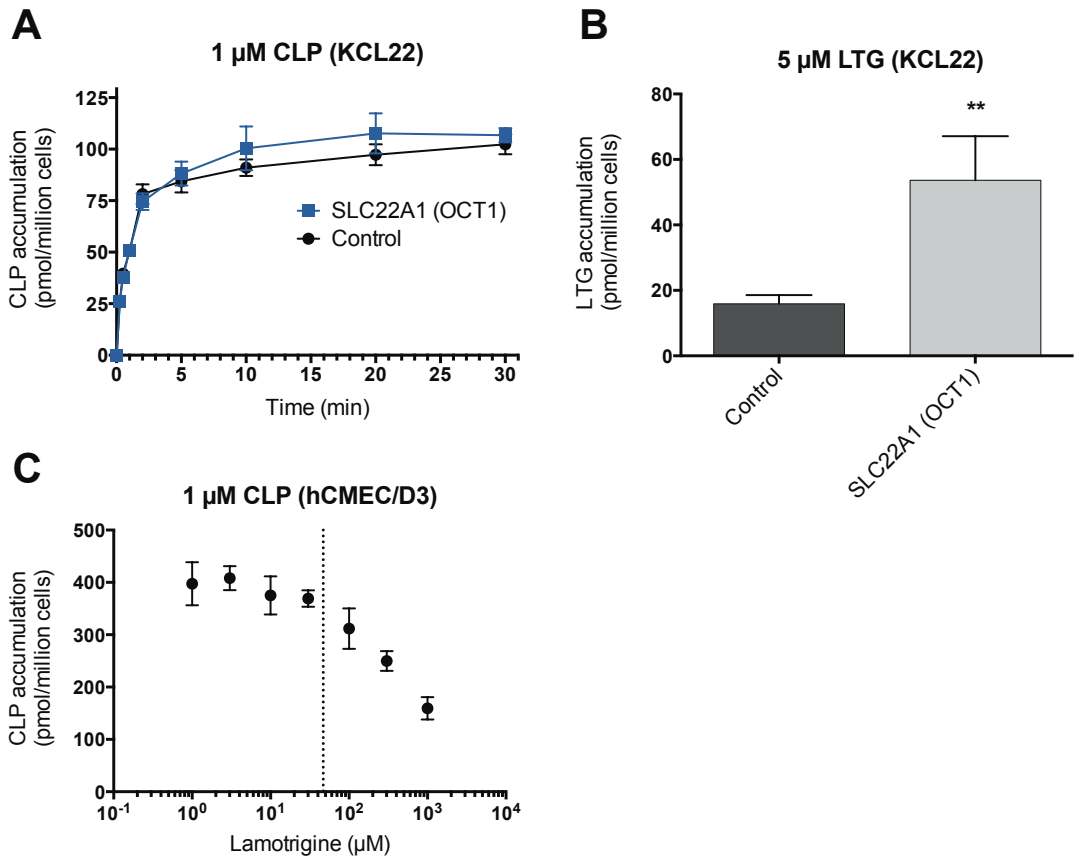


Figure 4.2: Analysis of clozapine as a potential SLC22A1 (OCT1) substrate and interactions with lamotrigine

A) Time-course of clozapine (CLP) uptake into KCL22 cells transfected with empty vector or *SLC22A1* at 37 °C; B) Positive control with uptake of lamotrigine (LTG) into KCL22 cells transfected with empty vector or *SLC22A1* after 30 minutes at 37 °C; C) Accumulation of CLP in the hCMEC/D3 cell line after 30 minutes at 37 °C in the presence of increasing LTG concentrations (1 – 1000 µM). The therapeutically relevant maximum LTG concentration of 47 µM is indicated by a dotted line; Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with ** for $p<0.01$

4.3.3. Interactions between lamotrigine and clozapine mediated by SLC22A1 (OCT1)

Based on the results obtained in section 4.3.2, the hypothesis that CLP effectiveness is enhanced in the presence of LTG by means of an SLC22A1 (OCT1) interaction had to be rejected. Unrelated to this hypothesis, however, CLP exhibits typical SLC22A1 inhibitor characteristics with a positive net charge at physiological pH and high lipophilicity (Ahlin *et al.* 2008). In addition, CLP has been reported to inhibit the SLC22A1-mediated uptake of 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP^+) by 47.5 % (Ahlin *et al.* 2008).

To test the effect of CLP on LTG transport, KCL22 cells, either transfected with empty vector or *SLC22A1*, were incubated with 5 µM LTG and 2.73 µM TEA^+, respectively, in the presence of increasing CLP concentrations ranging from 0.1 – 100 µM (Figure 4.3 A+B). TEA^+ is a SLC22A1 model substrate and served as positive control. A dose-response was observed for increasing CLP concentrations and curve-fitting yielded IC_{50} values of 1.8 µM (LTG as a substrate) and 5.7 µM (TEA^+ as a substrate), respectively. The analysis was extended to the hCMEC/D3 cell line, again with 5 µM LTG as a substrate and increasing CLP concentrations ranging from 0.1 – 100 µM (Figure 4.3 C). As before, a dose-response was observed and curve-fitting resulted in a similar IC_{50} of 2.0 µM. These *in vitro* results indicate that CLP can potently inhibit SLC22A1-mediated LTG transport at therapeutically relevant concentrations.

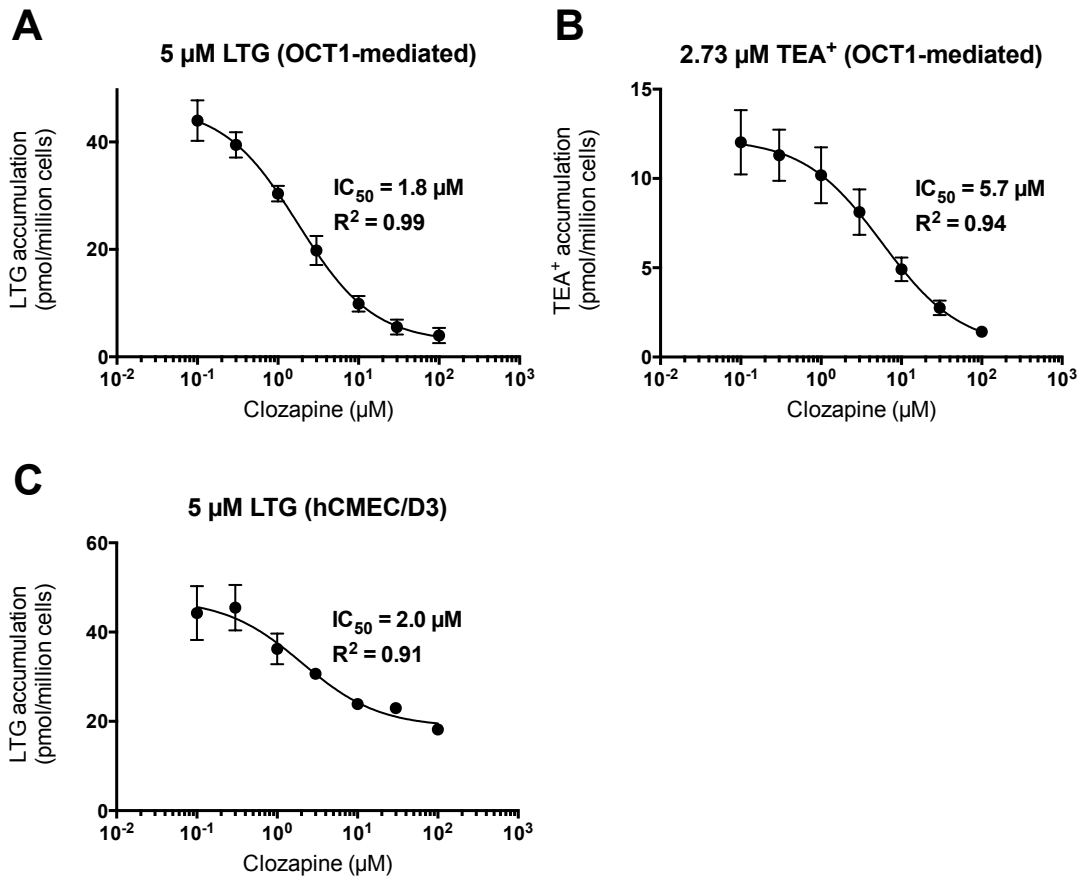


Figure 4.3: Drug-drug interactions between lamotrigine and clozapine

Uptake of LTG (A) and TEA^+ (B) into KCL22 cells transfected with empty vector or *SLC22A1,* determined after 30 minutes at 37 °C in the presence of increasing clozapine concentrations (0.1 – 100 µM). SLC22A1 (OCT1)-mediated uptake was calculated by subtracting the data derived from empty vector-transfected from *SLC22A1*-transfected KCL22 cells; C) Accumulation of LTG in the hCMEC/D3 cell line after 30 minutes at 37 °C in the presence of increasing clozapine concentrations (0.1 – 100 µM); All results are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). LTG = lamotrigine, TEA^+ = tetraethylammonium

To further characterise the interaction between LTG and CLP and its potential clinical relevance, additional assays were carried out as before but with primary human and rat hepatocytes (Figure 4.4). Primary human hepatocytes were extracted from healthy liver tissue donated from two patients that underwent surgery for easily resectable primary hepatocellular carcinoma or colorectal liver metastases. The baseline clinical and demographic characteristics are summarised in Table 4.1. A dose-response was observed with all three primary cell lines and

curve-fitting yielded IC_{50} values of 7.9 µM for patient 1, 3.9 µM for patient 2, and 4.7 µM for rat hepatocytes (Figure 4.4). Cell viability after hepatocyte extraction was 79 % for patient 1, 89 % for patient 2, and 90 % for rat. The viability of hepatocytes derived from patient 1 was slightly below the threshold of 85 % but considered acceptable because of the high value of these cells.

Table 4.1: Baseline clinical and demographic characteristics for patients who donated hepatocytes

	Patient 1	**Patient 2**
Sex	Male	Male
Age (years)	65	75
Weight (kg)	72	67
Race	Caucasian	Caucasian
Smoking status	Non-smoker	Non-smoker
Alcohol consumption	Zero	Zero
Underlying disease	Colorectal liver metastasis	Unknown

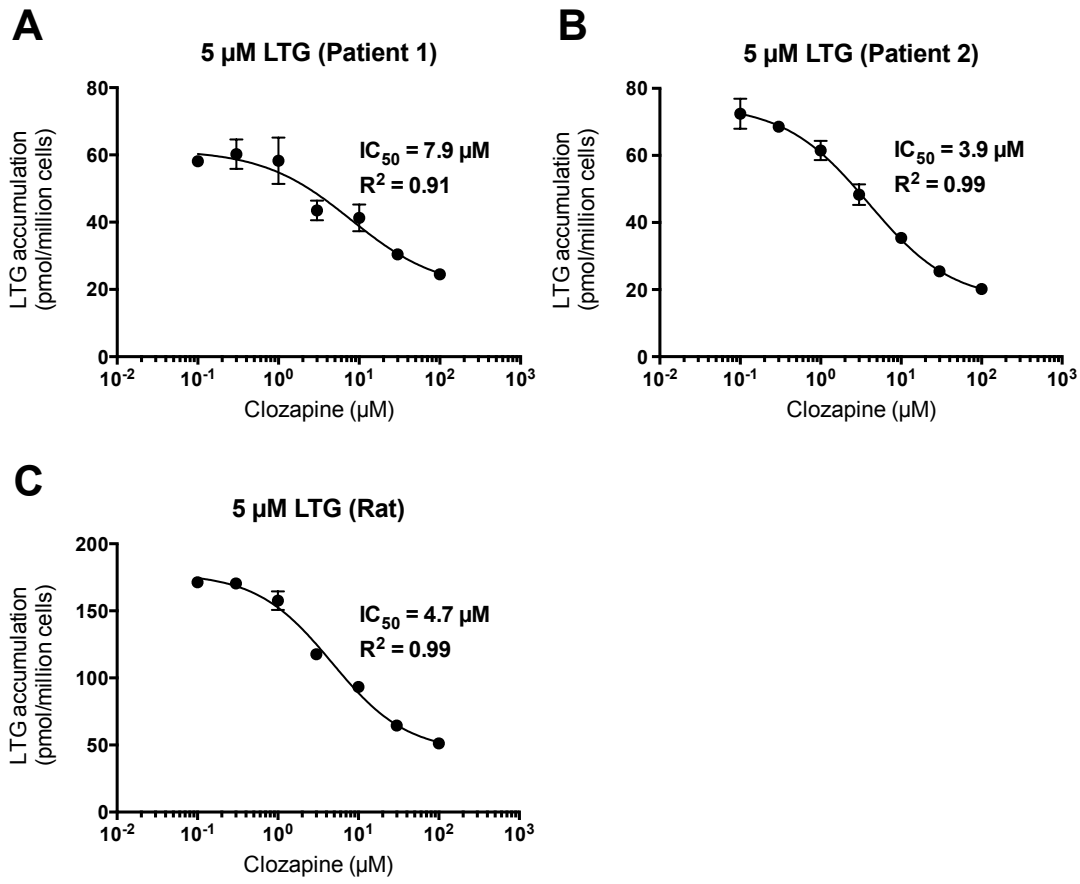


Figure 4.4: Drug-drug interactions between lamotrigine and clozapine in primary human and rat hepatocytes

Accumulation of lamotrigine (LTG) after 30 minutes at 37 °C in the presence of increasing clozapine concentrations (0.1 – 100 µM); A) Primary human hepatocytes from patient 1; B) Primary human hepatocytes from patient 2; C) Primary rat hepatocytes; All results are expressed as means ± standard deviation (n = 1 experiment in triplicate)

SLC22A1 gene expression levels in primary human hepatocytes and the hCMEC/D3 cell line were analysed by TaqMan® real-time PCR using 100 ng cDNA. Initially, *GAPDH* was utilised as the housekeeping gene for normalisation. However, while the C_t for *SLC22A1* gene expression was 29 for both patients, the C_t obtained for *GAPDH* gene expression was dramatically different with only 36 for patient 2 but 26 for patient 1. Assuming a primer efficacy of 2, this corresponds to a 1024-fold difference in *GAPDH* gene expression. The underlying reason for this observation is unknown but might be linked to the cancer in the patient. In any case, *GAPDH* is not

suitable for normalisation and the assay was therefore repeated using beta-actin (*ACTB*) as an alternative housekeeping gene. In contrast to *GAPDH*, the C_t for the *ACTB* gene expression was 26 for both human hepatocyte batches. In addition, the C_t for *SLC22A1* was 30 for both patients. Surprisingly, *SLC22A1* was only borderline expressed in the hCMEC/D3 cell line with a C_t of 39. The relative *SLC22A1* mRNA expression levels, normalised to *ACTB*, are summarised in Figure 4.5 and plotted as fold difference to the expression in the hCMEC/D3 cell line. Hepatocytes from both patients displayed on average 915-fold higher *SLC22A1* mRNA expression than hCMEC/D3 cells.

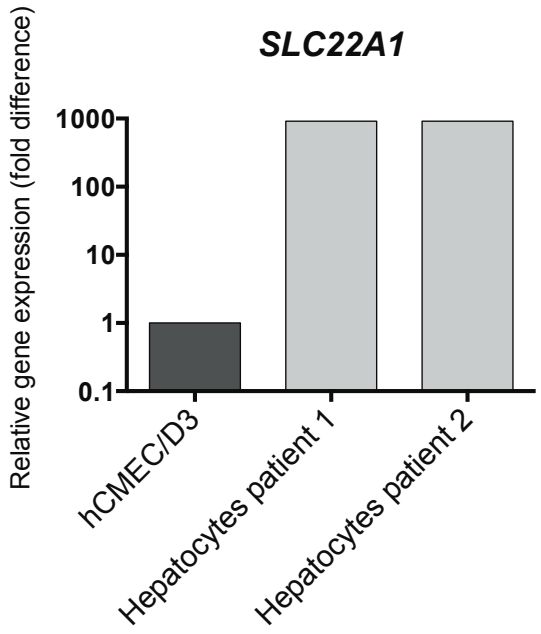


Figure 4.5: Relative gene expression of *SLC22A1* in primary human hepatocytes and hCMEC/D3 cells

Gene expression of *SLC22A1* in two batches of primary human hepatocytes relative to the expression in hCMEC/D3 cells normalised to beta-actin (*ACTB*). The relative gene expression is given as fold difference. Data are expressed as means and were derived from 1 experiment analysed in quadruplicate

4.4. Discussion

Numerous drugs are known to inhibit SLC22A1 (OCT1) and the risk for drug-drug interactions with LTG as an SLC22A1 substrate is high. From the literature, two clinical interactions were noted and investigated *in vitro*, an interaction between LTG and the HIV protease inhibitors lopinavir/ritonavir, and a potential interaction between LTG and the antipsychotic clozapine.

Lopinavir/ritonavir have been reported to reduce LTG serum levels by about 50 %. To analyse a potential interaction at the drug transporter level, the accumulation of LTG was assessed in empty vector-transfected and *SLC22A1*-transfected KCL22 cells in the presence of several antiretroviral drugs. At therapeutically relevant concentrations, a combination of lopinavir/ritonavir significantly reduced the SLC22A1-mediated uptake of LTG by 39 %. The same 39 % reduction was observed with lopinavir alone, suggesting that lopinavir, at clinically relevant concentrations, is the dominant or sole inhibitor of SLC22A1-mediated LTG uptake.

Ritonavir has previously been described as an SLC22A1 inhibitor utilising the two model substrates TEA^+ and MPP^+ (Jung *et al.* 2008, Jung *et al.* 2012, Zhang *et al.* 2000). This observation could be confirmed for SLC22A1-mediated LTG uptake using ritonavir at a concentration of 10 µM. However, 10 µM is more than one order of magnitude higher than relevant peak plasma levels when ritonavir is used as a pharmacokinetic booster (AbbVie Inc. 2013). The inhibitory characteristics of lopinavir were further investigated utilising a range of concentrations from 0.1 – 100 µM. A dose-response curve was difficult to fit due to the slowly increasing inhibitory potency combined with a high SD, likely a technical consequence of the non-adherent KCL22 cell lines that required extensive washing with centrifugation between each washing step. In addition, the curve appears to reach a plateau and may thus describe a biphasic dose-response. However, many more repeats and data points with higher and lower concentrations would be required to gain confidence in this hypothesis. Notably, a biphasic dose-response has been described before for

the inhibition of SLC22A1-mediated MPP^+ uptake by lamivudine, concluding that SLC22A1 has a low-affinity and high-affinity binding site (Minuesa *et al.* 2009). The same study also found abacavir to be a very potent inhibitor of MPP^+ uptake. Interestingly, lamivudine and abacavir both had no effect on LTG uptake as presented here, suggesting that LTG may bind to different site(s) than MPP^+ within the substrate-binding cavity of SLC22A1.

In conclusion, this is the first study reporting an inhibition of SLC22A1-mediated LTG uptake by lopinavir at clinically relevant concentrations. Reduced LTG serum levels in the presence of lopinavir/ritonavir have been reported before and were correlated to an increased LTG metabolism (AbbVie Inc. 2013, Burger *et al.* 2008, GlaxoSmithKline Inc. 2012, van der Lee *et al.* 2006). The data presented here suggest an additional effect from an interaction with SLC22A1, for example through reduced LTG uptake from the intestine (Han *et al.* 2013).

LTG may be beneficial as an augmentation strategy to overcome CLP resistance for the treatment of schizophrenia, but no mechanistic pharmacological studies have been done so far. To analyse a potential interaction at the drug transporter level, the accumulation of CLP was assessed in empty vector-transfected and *SLC22A1*-transfected KCL22 cells in a time-course assay up to 30 minutes. No difference was observed indicating that CLP is not a substrate for SLC22A1. Thus, based on these data, the initial hypothesis had to be rejected. CLP, however, may still act as an inhibitor of SLC22A1 as it exhibits typical SLC22A1 inhibitor characteristics (Ahlin *et al.* 2008). An impaired SLC22A1-mediated LTG uptake by means of CLP could be clinically important because CLP treatment is associated with an elevated risk for seizures (boxed warning) (Novartis Pharmaceuticals Corporation 2013). The drug of choice for the treatment of CLP-induced seizures is VPA, but LTG is increasingly recognised as an alternative (Varma *et al.* 2011).

The accumulation of LTG was analysed in the presence of increasing CLP concentrations utilising different cell lines. First, empty vector-transfected and *SLC22A1*-transfected KCL22 cells yielded an SLC22A1-mediated inhibition with an

IC_{50} as low as 1.8 µM. A very similar effect was seen in the hCMEC/D3 cell line as an *in vitro* model of the BBB yielding an IC_{50} of 2.0 µM. These numbers are within therapeutically relevant CLP concentration ranges of 0.3 µM to 2.4 µM (Novartis Pharmaceuticals Corporation 2013). Primary human and rat hepatocytes further confirmed the observed interaction. Experiments with hepatocytes derived from two patients yielded IC_{50} values of 7.9 µM and 3.9 µM, respectively. The latter value might be more reliable as the hepatocytes from patient 1 showed a viability of only 79 % and the experimental data had larger SDs. In addition, the R^2 as a measure of the goodness of fit was 0.91 (patient 1) vs. 0.99 (patient 2). Rat hepatocytes yielded an IC_{50} of 4.7 µM and R^2 of 0.99. Overall, a strong inhibitory effect of CLP was observed on the SLC22A1-mediated uptake of LTG in different cell lines including primary hepatocytes. The IC_{50} values were close together in the low, single-digit micromolar range at therapeutically relevant concentrations.

SLC22A1 is known to be most abundantly expressed in the liver (Nies *et al.* 2011) and hepatocytes derived from both patients showed strong and comparable *SLC22A1* mRNA expression levels. Surprisingly, the hCMEC/D3 cell line only exhibited a very borderline *SLC22A1* mRNA expression. While this corresponds to the lower effect size as compared to hepatocytes or *SLC22A1*-transfected KCL22 cells, it remains possible that, in addition to SLC22A1, LTG is a substrate for another, as yet unknown, drug transporter.

In conclusion, CLP potently inhibits SLC22A1-mediated LTG transport at therapeutically relevant concentrations. These *in vitro* observations might have consequences for the safety and/or effectiveness of LTG when co-administered with CLP. At the BBB, LTG-CLP interaction could impair the uptake of LTG and thus affect the drug´s effectiveness in the treatment of epilepsy or bipolar I disorder (Figure 4.6 C). In the liver, in contrast, an impaired LTG uptake into hepatocytes may increase the plasma concentration of LTG with an increased risk of side effects (Figure 4.6 B). In the intestine, a reduced LTG absorbance may result in lower oral bioavailability and consequently reduced efficacy (Figure 4.6 A). Finally, a reduced LTG reabsorption by tubule epithelial kidney cells may increase the drug´s clearance (Figure 4.6 D). The results obtained from this chapter with primary human

hepatocytes and the fact that SLC22A1 is most abundantly expressed in the liver suggests that impaired LTG uptake into hepatocytes may be of particular importance. The beneficial effects observed with co-treatment by CLP and LTG in treatment resistant schizophrenia could result from a net increase of LTG plasma levels. Rather than having a pharmacokinetic effect on CLP efficiency, LTG could have a therapeutic role and elevated LTG plasma levels may be of critical importance. It has been proposed before that LTG adds to or synergises the effect of CLP in treatment resistant schizophrenia by modulating neuronal glutamate transmission (Large *et al.* 2005). The potential clinical consequences that derive from the presented *in vitro* observations, however, require further research.

To assess whether a transporter other than SLC22A1 (OCT1) is involved in an interaction between CLP and LTG, the accumulation of CLP was studied in hCMEC/D3 cells in the presence of increasing LTG concentrations. No effect was observed up to the maximum therapeutic LTG plasma levels of 47 µM. Interestingly, a higher LTG concentration of ≥ 100 µM resulted in a decreased CLP accumulation, suggesting that an unknown transporter could be involved in CLP uptake. This is further supported by the observation that the uptake of 1 µM CLP (about 400 pmol/million cells after 30 minutes at 37 °C) is high when compared to all other dugs studied in this thesis. CLP uptake by an unidentified transporter will be the focus of chapter 5.

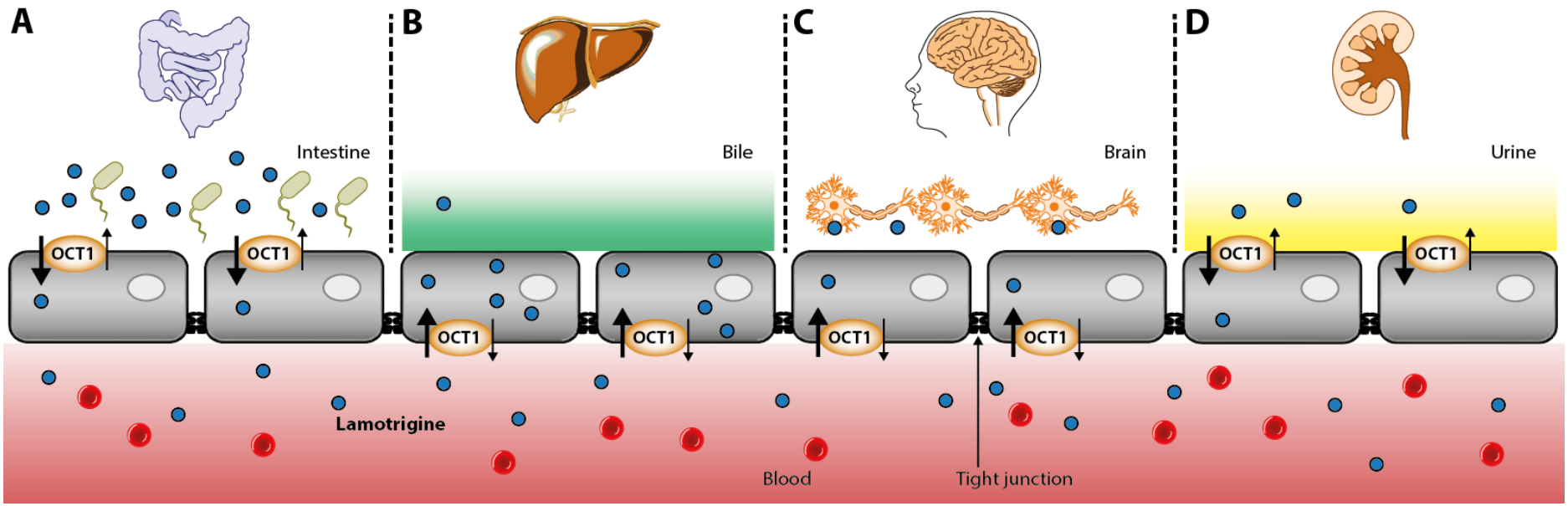


Figure 4.6: Illustration of a potential contribution of SLC22A1 (OCT1) to absorption, metabolism, distribution, and elimination (ADME) of lamotrigine

Simplified model illustrating the subcellular localisation of SLC22A1 (OCT1) at four pharmacologically important barriers potentially involved in lamotrigine absorption (epithelial cells of the intestine A), metabolism (liver hepatocytes B), distribution (brain endothelial cells C), and elimination (tubule epithelial cells of the kidney D)

4.5. References

AbbVie Inc. (2013) Kaletra Prescribing Information

Ahlin, G.*, et al.* (2008) Structural requirements for drug inhibition of the liver specific human organic cation transport protein 1. *J Med Chem* **51**, 5932-5942

Andersson, M. L., Bjorkhem-Bergman, L., and Lindh, J. D. (2011) Possible drug-drug interaction between quetiapine and lamotrigine--evidence from a Swedish TDM database. *Br J Clin Pharmacol* **72**, 153-156

Birbeck, G. L.*, et al.* (2012) Antiepileptic drug selection for people with HIV/AIDS: evidence-based guidelines from the ILAE and AAN. *Epilepsia* **53**, 207-214

Burger, D. M.*, et al.* (2008) The effect of atazanavir and atazanavir/ritonavir on UDP-glucuronosyltransferase using lamotrigine as a phenotypic probe. *Clinical pharmacology and therapeutics* **84**, 698-703

Castberg, I., Skogvoll, E., and Spigset, O. (2007) Quetiapine and drug interactions: evidence from a routine therapeutic drug monitoring service. *The Journal of clinical psychiatry* **68**, 1540-1545

Chakos, M.*, et al.* (2001) Effectiveness of second-generation antipsychotics in patients with treatment-resistant schizophrenia: a review and meta-analysis of randomized trials. *The American journal of psychiatry* **158**, 518-526

Dickens, D.*, et al.* (2012) Lamotrigine is a substrate for OCT1 in brain endothelial cells. *Biochemical pharmacology* **83**, 805-814

Giannoudis, A.*, et al.* (2008) Effective dasatinib uptake may occur without human organic cation transporter 1 (hOCT1): implications for the treatment of imatinib-resistant chronic myeloid leukemia. *Blood* **112**, 3348-3354

GlaxoSmithKline Inc. (2012) Lamictal Product Monograph

Goff, D. C.*, et al.* (2007) Lamotrigine as add-on therapy in schizophrenia: results of 2 placebo-controlled trials. *Journal of clinical psychopharmacology* **27**, 582-589

Han, T. K.*, et al.* (2013) Organic Cation Transporter 1 (OCT1/mOct1) is Localized in the Apical Membrane of Caco-2 Cell Monolayers and Enterocytes. *Molecular pharmacology*

Jung, N.*, et al.* (2008) Relevance of the organic cation transporters 1 and 2 for antiretroviral drug therapy in human immunodeficiency virus infection. *Drug metabolism and disposition: the biological fate of chemicals* **36**, 1616-1623

Jung, N.*, et al.* (2012) Organic cation transporters OCT1 and OCT2 determine the accumulation of lamivudine in CD4 cells of HIV-infected patients. *Infection*

Kane, J.*, et al.* (1988) Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. *Archives of general psychiatry* **45**, 789-796

Kremer, I.*, et al.* (2004) Placebo-controlled trial of lamotrigine added to conventional and atypical antipsychotics in schizophrenia. *Biological psychiatry* **56**, 441-446

Large, C. H., Webster, E. L., and Goff, D. C. (2005) The potential role of lamotrigine in schizophrenia. *Psychopharmacology* **181**, 415-436

Lieberman, J. A.*, et al.* (1994) Clinical effects of clozapine in chronic schizophrenia: response to treatment and predictors of outcome. *The American journal of psychiatry* **151**, 1744-1752

Minuesa, G.*, et al.* (2009) Transport of lamivudine [(-)-beta-L-2',3'-dideoxy-3'-thiacytidine] and high-affinity interaction of nucleoside reverse transcriptase inhibitors with human organic cation transporters 1, 2, and 3. *J Pharmacol Exp Ther* **329**, 252-261

National Institute for Health and Clinical Excellence (2012) The epilepsies: the diagnosis and management of the epilepsies in adults and children in primary and secondary care (NICE clinical guideline 137)

Nies, A. T.*, et al.* (2011) Organic Cation Transporters (OCTs, MATEs), In Vitro and In Vivo Evidence for the Importance in Drug Therapy. *Handb.Exp.Pharmacol.* **201**, 105-167

Novartis Pharmaceuticals Corporation (2013) Clozaril Prescribing Information

Porcelli, S., Balzarro, B., and Serretti, A. (2012) Clozapine resistance: augmentation strategies. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* **22**, 165-182

Sommer, I. E.*, et al.* (2012) Pharmacological augmentation strategies for schizophrenia patients with insufficient response to clozapine: a quantitative literature review. *Schizophrenia bulletin* **38**, 1003-1011

Sondergaard Khinchi, M.*, et al.* (2008) Lamotrigine therapeutic thresholds. *Seizure : the journal of the British Epilepsy Association* **17**, 391-395

Tiihonen, J.*, et al.* (2003) Lamotrigine in treatment-resistant schizophrenia: a randomized placebo-controlled crossover trial. *Biological psychiatry* **54**, 1241-1248

Tiihonen, J., Wahlbeck, K., and Kiviniemi, V. (2009) The efficacy of lamotrigine in clozapine-resistant schizophrenia: a systematic review and meta-analysis. *Schizophrenia research* **109**, 10-14

van der Lee, M. J.*, et al.* (2006) Lopinavir/ritonavir reduces lamotrigine plasma concentrations in healthy subjects. *Clinical pharmacology and therapeutics* **80**, 159-168

Varma, S.*, et al.* (2011) Clozapine-related EEG changes and seizures: dose and plasma-level relationships. *Therapeutic advances in psychopharmacology* **1**, 47-66

Zhang, L.*, et al.* (2000) Interactions of HIV protease inhibitors with a human organic cation transporter in a mammalian expression system. *Drug metabolism and disposition: the biological fate of chemicals* **28**, 329-334

Zoccali, R.*, et al.* (2007) The effect of lamotrigine augmentation of clozapine in a sample of treatment-resistant schizophrenic patients: a double-blind, placebo-controlled study. *Schizophrenia research* **93**, 109-116

Chapter 5

Clozapine uptake into the brain

Contents

5.1. Introduction

The results from chapter 4 of this thesis suggest that an unidentified uptake transporter may be involved in CLP accumulation in the hCMEC/D3 cell line. CLP is an important last-resort treatment option for patients suffering from otherwise treatment-resistant schizophrenia (Novartis Pharmaceuticals Corporation 2013).

Schizophrenia is a severe mental illness affecting about 24 million people worldwide (World Health Organisation 2014). It is characterised by symptoms of altered perception, thought, affect, and behaviour (National Institute for Health and Clinical Excellence 2009). Schizophrenia can have a devastating effect on a patient's social life. The disease is accompanied by increased mortality, leading to reduced life expectancy of 12 – 15 years as compared to the general population (National Institute for Health and Clinical Excellence 2009, van Os *et al.* 2009).

Pathophysiologically, schizophrenia is associated with elevated dopamine synthesis, release, and elevated synaptic concentrations at resting-state (van Os *et al.* 2009). Consequently, all currently approved treatments interfere with the dopaminergic circuit and the dopamine D_2 receptor is regarded as the main target (Kapur *et al.* 2006, Miyamoto *et al.* 2012, van Os *et al.* 2009). However, most antipsychotic drugs (APDs) also affect other neurotransmitter receptors such as serotonergic, adrenergic, and/or muscarinic receptors. In addition, monoamine transporters from the SLC6A family are frequently affected (Miyamoto *et al.* 2012). The action on multiple but still distinct targets is assumed to define the overall therapeutic and side effect profile of each APD (Miyamoto *et al.* 2012).

APDs are classified as first-generation APDs (FGD) or second-generation APDs (SGD). SGDs, with its prototypical drug clozapine, are also referred to as atypical APDs (Miyamoto *et al.* 2012). The term "atypical" derives from the observation that treatment with SGDs is associated with reduced occurrence of extrapyramidal side effects, a common problem with FGDs that has been linked to the high D_2 receptor occupancy (Miyamoto *et al.* 2012). High affinity for the serotonin receptor 5-HT_{2A} in combination with reduced, but still present, occupancy

of D_2 is thought to be the underlying molecular reason leading to the "atypical" behaviour of SGDs (Meltzer *et al.* 1989, Miyamoto *et al.* 2012).

Pharmacological treatment is of fundamental importance to cope with the symptoms of schizophrenia. However, about one third of all patients do not respond adequately to standard treatment options and remain refractory (van Os *et al.* 2009). In the UK, a patient is classified as refractory if treatment with at least two APDs, including one SGD (except CLP), has failed (National Institute for Health and Clinical Excellence 2009). CLP is approved for the treatment of schizophrenia in otherwise refractory patients and has demonstrated clear superiority to chlorpromazine with a response rate of 30 % vs. 4 % (Kane *et al.* 1988, Novartis Pharmaceuticals Corporation 2013). Based on this study, Novartis received FDA marketing authorisation for CLP in 1989.

CLP is about 97 % serum-protein bound and reaches average steady-state peak plasma concentrations of about 1 µM (range from 0.3 – 2.4 µM) following twice-daily doses of 100 mg (Novartis Pharmaceuticals Corporation 2013). The drug is extensively and almost completely metabolised to three principal CLP metabolites, namely desmethylclozapine (norclozapine), clozapine N-oxide, and hydroxyclozapine (Novartis Pharmaceuticals Corporation 2013). While norclozapine has been reported to show some limited pharmacological activity, the latter two are inactive (Novartis Pharmaceuticals Corporation 2013). The main enzymes involved in CLP metabolism are CYP1A2 and CYP3A4, which does lead to critical drug-drug interactions (Eiermann *et al.* 1997, Pirmohamed *et al.* 1995).

As yet, CLP has not been demonstrated to be a substrate for any particular human drug transporter using direct approaches. The transepithelial transport of tritium-labelled CLP across a human *ABCB1*-transfected pig epithelial kidney cell line was negative (Schinkel *et al.* 1996). In contrast, several studies have found an inhibitory effect on the uptake or efflux of model substrates for SLC22A1 (OCT1), SLC22A2 (OCT2), SLC22A3 (OCT3), SLC29A4 (ENT4), ABCB1 (MDR1, Pgp), and ABCG2 (BCRP) using overexpressing cell lines (Ahlin *et al.* 2008, Haenisch *et al.* 2010, Haenisch *et al.* 2012, Schmitt *et al.* 2012, Wang *et al.* 2006, Wang *et al.* 2008).

However, most of the reported IC_{50} values were considerably above average CLP peak plasma levels of 1 µM. Interestingly, the lowest IC_{50} value of 6.65 µM was reported for SLC22A1 using 15 nM MPP^+ as a model substrate (Haenisch *et al.* 2012) (see also chapter 4). *In vivo*, the mouse *Abcb1a/Abcb1b* (-/-) knockout model was used to elicit a potential effect on CLP brain to serum/plasma AUC concentration ratios. While one study reported no effect (Schmitt *et al.* 2012), another found a minor difference as compared to WT animals with a brain to plasma AUC ratio of 6.6 vs. 4.1 (Doran *et al.* 2005).

The reason for CLP only being licensed as a reserve treatment option is the substantially increased risk for agranulocytosis and seizures (boxed warnings) (Novartis Pharmaceuticals Corporation 2013). Due to the risk for agranulocytosis, an extensive blood monitoring (weekly for the first six months) is required for every patient treated with the drug. Other boxed warnings include myocarditis, cardiomyopathy, orthostatic hypotension, bradycardia, syncope, and an increased mortality in elderly patients with dementia-related psychosis (Novartis Pharmaceuticals Corporation 2013).

In view of these severe adverse effects with unknown aetiology, transporter-mediated cellular uptake of CLP could be a critical component. Additional evidence for this hypothesis is present in the literature with two studies of particular note. First, likely transporter-mediated, active CLP uptake into the promyelocytic leukemia cell line HL-60 has been described (Henning *et al.* 2002). The second study reported eight times higher CLP concentrations in leukocytes derived from a patient with CLP-induced agranulocytosis compared with ten patients treated with CLP but no evidence of agranulocytosis (Bergemann *et al.* 2007). After correcting for CLP plasma levels a difference of about six was noted.

Theoretically, variants or differences in expression levels of this unknown transporter might predict the effectiveness (e.g. uptake into the brain) and/or adverse effects (e.g. uptake into leukocytes, cardiomyocytes, or brain) of CLP, allowing personalised prescription. The aim of this research chapter was therefore

to identify and characterise the unknown CLP uptake transporter as hypothesised based on data from chapter 4 and the literature.

5.2. Materials and methods

5.2.1. Materials

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich Company Ltd., Gillingham, UK, or listed in the corresponding sections of chapter 3 and 4 (3.2.1 and 4.2.1). Carbon-14-labelled TEA^+ ($[^{14}C]$-TEA^+, 0.1 mCi/ml, specific activity 3.5 mCi/mmol) was obtained from PerkinElmer, Seer Green, UK.

5.2.2. Cell lines and culture conditions

5.2.2.1. hCMEC/D3 cell line

Please refer to chapter 3 section 3.2.2.1.

5.2.2.2. KCL22 cell line

The human chronic myeloid leukaemia cell line KCL22 was stably transfected with an empty vector (control) or a vector encoding for SLC22A4 (OCTN1) by means of electroporation. Both non-adherent cell lines were a kind gift from Dr Athina Giannoudis (Department of Haematology, University of Liverpool, UK) and cultured in RPMI-1640 medium supplemented with 10 % FBS (v/v) and 1 % penicillin-streptomycin (v/v) at 37 °C and 5 % CO_2 (Dickens *et al.* 2013a).

5.2.3. Distribution coefficient (logD, pH 7.4)

The distribution coefficient (logD, pH 7.4) was determined as outlined in chapter 3 section 3.2.3.

5.2.4. Drug accumulation assays

5.2.4.1. Non-adherent cells

Cell accumulation assays with non-adherent KCL22 cells, stably transfected with an empty vector or *SLC22A4*, were carried out as described in chapter 4 section 4.2.3.1. The double-concentrated master mix consisted of transport buffer (HBSS supplemented with 25 mM HEPES, adjusted to pH 7.4) with 1.2 µCi/ml [^{14}C]-TEA$^+$ or 0.2 µCi/ml [^{3}H]-CLP, non-labelled corresponding "cold" CLP to give 2 µM, and 0.2 % BSA (w/v). The final vehicle concentration did not exceed 0.6 % per reaction (Ethanol and/or DMSO). The positive control with [^{14}C]-TEA$^+$ did not contain any additional "cold" drug and resulted in a final concentration of 171.42 µM TEA$^+$.

5.2.4.2. Adherent cells

Cell accumulation assays with adherent hCMEC/D3 cells were carried out as described in chapter 3 section 3.2.4.

The master mix for CLP time-course assays, siRNA screenings, and assays to determine the effect of temperature consisted of transport buffer (HBSS supplemented with 25 mM HEPES, adjusted to pH 7.4) with 0.1 µCi/ml [^{3}H]-CLP, non-labelled corresponding "cold" CLP to give a final concentration of 1 µM, and 0.1 % BSA (w/v). The vehicle concentration did not exceed 0.2 % per reaction (DMSO).

The master mix for inhibitor screenings consisted of transport buffer with 0.1 µCi/ml [^{3}H]-CLP, non-labelled corresponding "cold" CLP to give a final concentration of 1 µM, the inhibitor or vehicle only as control, and 0.1 % BSA (w/v). The vehicle concentration did not exceed 0.6 % per reaction (DMSO and HCl). Norepinephrine was dissolved in 1 M HCl with no effect on the pH of the master mix.

The master mix to determine CLP uptake kinetics consisted of transport buffer with 0.05 µCi/ml [^{3}H]-CLP, non-labelled corresponding "cold" CLP to give final

concentrations ranging from 0.1 – 300 µM, and 0.1 % BSA (w/v). The vehicle concentration did not exceed 0.4 % per reaction (DMSO).

5.2.5. siRNA transfection

RNAi experiments were carried out as outlined in chapter 3 section 3.2.5. All siRNA sequences and corresponding identification numbers are listed in the appendix.

5.2.6. Gene expression

RNA extraction, RT-PCR, and real-time PCR were carried out as described in chapter 3 section 3.2.6. TaqMan® assay probe sequences and identification numbers are listed in the appendix.

5.2.7. Statistical analysis

All data are presented as means ± SD. One-way ANOVA followed by Dunnett's post-hoc test was carried out for statistical analysis of all experiments with multiple comparisons. Single comparisons were analysed by an independent, two-tailed t-test. Significant results are indicated with * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$. Analysis was performed with SPSS® Statistics version 20 (IBM United Kingdom Ltd., Hampshire, UK). Kinetic parameters were calculated with Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) by fitting a non-linear Michaelis-Menten regression curve to the data.

5.3. Results

5.3.1. Lipophilicity of clozapine

CLP is a lipophilic drug with a predicted logD (pH 7.4) of 1.95 (ACD labs algorithm on www.chemspider.com). Here, a slightly lower experimental logD (pH 7.4) of 1.36 ± 0.01 was obtained. Sugano *et al.* referred to drugs with a logD between 0 – 2 as having moderate lipophilicity (Sugano *et al.* 2010). A previous study using a similar experimental approach reported a logD (pH 7.4) of 2.7 (Hartter *et al.* 2003). However, despite some differences between the absolute numbers, all three predicted and experimentally derived logDs (pH 7.4) were within the range of 1 – 4, an area that has been described as optimal for passive diffusion across the BBB (van de Waterbeemd *et al.* 1998). Therefore, an active uptake of CLP has not received much attention but there is growing evidence, including data from this thesis (chapter 4), that CLP could be a substrate for an unidentified uptake transporter.

5.3.2. Time-course of clozapine uptake into the hCMEC/D3 cell line and the effect of temperature

To investigate potential carrier-mediated CLP uptake into hCMEC/D3 cells, a time-course assay was carried out (data obtained at 0.15, 0.5, 1, 2, 5, 10, 20, and 30 minutes) and the effect of temperature was investigated. CLP uptake into hCMEC/D3 cells exhibited typical transporter kinetics with a linear phase of uptake (about two minutes) that was saturable (Figure 5.1 A). In addition, lowering the temperature to 4 °C was accompanied by significantly decreased CLP accumulation after 30 minutes (Figure 5.1 B). Both results support the involvement of an active CLP uptake transporter.

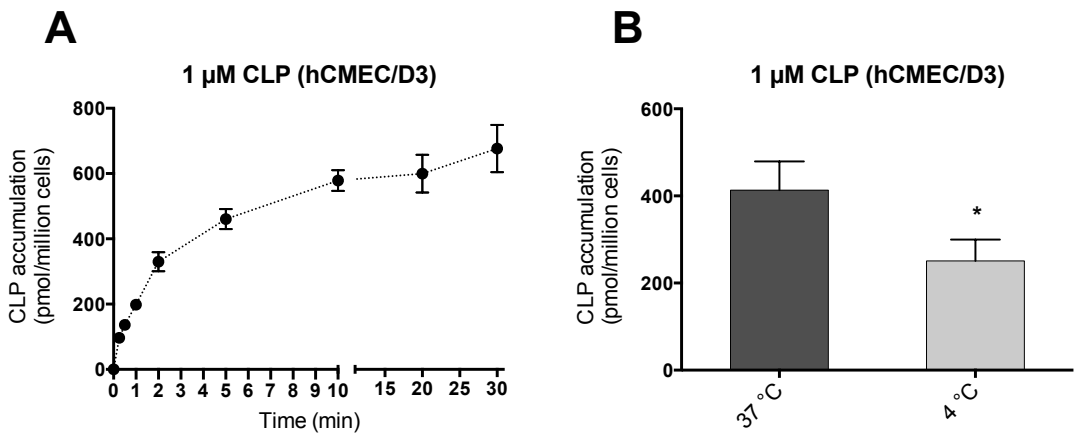


Figure 5.1: Time-course of clozapine uptake and the effect of temperature

A) Time-course accumulation assay of clozapine (CLP) in hCMEC/D3 cells at 37 °C. Data were obtained at 0.15, 0.5, 1, 2, 5, 10, 20, and 30 minutes, respectively; B) Accumulation of clozapine in hCMEC/D3 cells after 30 minutes at 37 °C and 4 °C, respectively; All data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with * for $p<0.05$

5.3.3. Initial chemical inhibitor screening to characterise the uptake of clozapine into the hCMEC/D3 cell line

Chemical inhibitor screening was applied as a first step to identify the transporter class potentially responsible for CLP uptake into hCMEC/D3 cells. The selected compounds (including substrates as competitive inhibitors) are listed in Table 5.1 with corresponding typical and important drug transporter classes affected. The term “class” shall denote a drug transporter family, subfamily, or further sub-divisions such as the OATs or OCTs within the SLC22A subfamily (chapter 1 section 1.2.1.3). Chemical inhibitors can never be considered as absolutely specific and thus a transporter class was regarded as affected when one or more transporters have been shown to interact with the designated compound. This should not imply, however, that every member from within these transporter classes is necessarily affected. In chapter 4 section 4.3.2 LTG was shown to inhibit CLP uptake with concentrations ≥ 100 µM. This was included as a positive control.

The accumulation of 1 µM CLP was determined separately in the presence of all selected chemical inhibitors after 30 minutes at 37 °C (Figure 5.2 A). Prazosin and verapamil were the most potent inhibitors, resulting in a reduced average CLP accumulation of 94 % and 83 %, respectively. The presence of LTG resulted in reduced average CLP accumulation of 26 %, expected based on the results from chapter 4 showing that LTG has inhibitory potencies beginning at concentrations of about 100 µM. Tariquidar exhibited a minor but significant effect on CLP accumulation with an average 15 % reduction.

Table 5.1: Selected chemical drug transporter inhibitors for initial screening to characterise the uptake of clozapine into the hCMEC/D3 cell line

Compound	Concentration	Affected drug transporter classes	References
Prazosin	100 µM	OCTs, ABCGs	(Matsson *et al.* 2007, Matsson *et al.* 2009, Nies *et al.* 2011)
Verapamil	100 µM	ABCBs, ABCGs, OCTs, OCTNs	(Giacomini *et al.* 2010, Matsson *et al.* 2009, Nies *et al.* 2011, Ohashi *et al.* 1999, Yabuuchi *et al.* 1999)
Lamotrigine	100 µM	OCTs	(Dickens *et al.* 2012)
Tariquidar	1 µM	ABCBs, ABCCs, ABCGs	(Dickens *et al.* 2013b, Kannan *et al.* 2011, Mistry *et al.* 2001, Sun *et al.* 2013)
PSC-833	10 µM	ABCBs	(Boesch *et al.* 1991, Dickens *et al.* 2013b)
Ko143	1 µM	ABCGs	(Allen *et al.* 2002)
MK571	50 µM	ABCCs, OATPs	(Keppler 2011, Letschert *et al.* 2005)
Indomethacin	100 µM	ABCCs, OATs, OCTs	(Burckhardt *et al.* 2011, Khamdang *et al.* 2002, Reid *et al.* 2003)
Methotrexate	100 µM	OATPs, OATs, ABCCs, ABCGs	(Badagnani *et al.* 2006, Burckhardt *et al.* 2011, Chen *et al.* 2003, Keppler 2011)

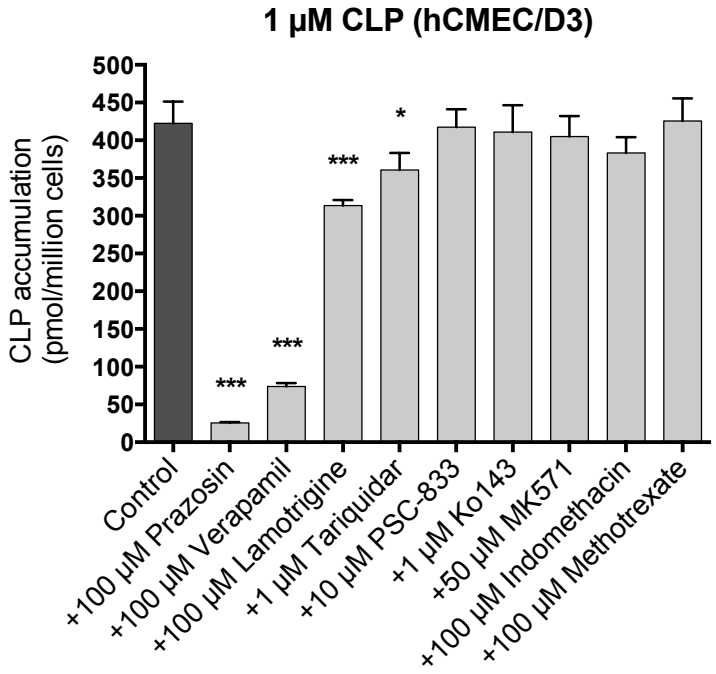


Figure 5.2: Initial chemical inhibitor screening to characterise the uptake of clozapine into the hCMEC/D3 cell line

Accumulation of clozapine (CLP) in the hCMEC/D3 cell line in the presence of various drug transporter inhibitors after 30 minutes at 37 °C. Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with * for $p<0.05$ and *** for $p<0.001$

5.3.4. Kinetics of clozapine uptake into the hCMEC/D3 cell line

The results from the initial inhibitor screening suggested that a strong uptake process was responsible for CLP accumulation in the hCMEC/D3 cell line. The kinetics were determined at 37 °C in the linear phase of uptake at a fixed time-point of one minute with increasing CLP concentrations ranging from 0.1 – 300 µM. CLP uptake followed nearly perfect Michaelis-Menten kinetics and curve fitting yielded an R^2 of 0.99, V_{max} of 3299 pmol/million cells, and K_m of 35.93 µM (Figure 5.3). Considering that average CLP peak plasma concentrations are around 1 µM, a K_m of 35.93 µM indicates a high-capacity CLP uptake transporter at therapeutically relevant concentrations.

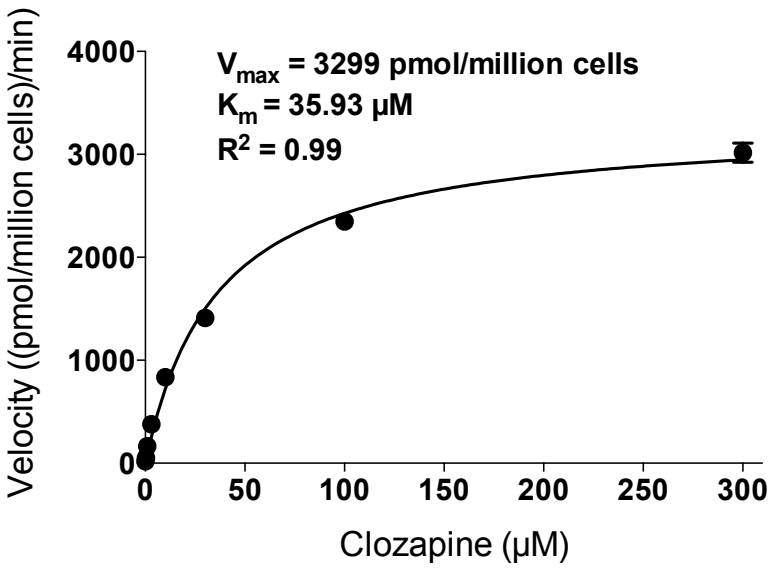


Figure 5.3: Michaelis-Menten kinetics of clozapine uptake into the hCMEC/D3 cell line

Clozapine uptake into hCMEC/D3 cells was determined after 1 minute at 37 °C with clozapine concentrations ranging from 0.1 – 300 µM. A Michaelis-Menten regression curve was fitted to the data and kinetic parameters V_{max} and K_m calculated. Data are expressed as means ± standard deviation (n = 6 independent experiments in triplicate)

5.3.5. Second chemical inhibitor screening to investigate SLC22A3 (OCT3) as a potential clozapine transporter

In chapter 4 section 4.3.3 CLP was shown to be a potent inhibitor of SLC22A1 (OCT1)-mediated LTG and TEA^+ transport. In addition, CLP is positively charged at physiological pH (Ahlin *et al.* 2008) and the inhibitor profile from the initial screening experiment further suggests that an organic cation transporter from the SLC22A transporter family may be involved in CLP uptake. SLC22A1, however, was excluded as a potential CLP uptake transporter in chapter 4 (section 4.3.2). Additionally, *SLC22A2* mRNA levels are not detectable in the hCMEC/D3 cell line (Carl *et al.* 2010, Dickens *et al.* 2012). However, the same two studies reported detectable *SLC22A3* mRNA levels encoding for the extraneuronal monoamine transporter SLC22A3 (OCT3).

Monoamines are a class of molecules that include important neurotransmitters such as dopamine, serotonin, and norepinephrine. Two monoamine transporter systems are distinguished in the literature and referred to as uptake$_1$ and uptake$_2$. Uptake$_1$ consists of high-affinity neuronal monoamine transporters, mainly SLC6A2-4 (NET, DAT, and SERT), while uptake$_2$ consists of high-capacity extraneuronal monoamine transporters, particularly SLC22A3 and SLC29A4 (ENT4) (Duan *et al.* 2010). At this stage, SLC22A3 was considered the most promising transporter candidate for CLP and could explain the observed high-capacity uptake. Additional screening was thus applied with several known SLC22A3 inhibitors to gain more confidence in this hypothesis. The selected inhibitors are listed in Table 5.2 and verapamil was included as a positive control. While verapamil again potently inhibited CLP uptake, none of the other inhibitors had an effect after 30 minutes at 37 °C (Figure 5.4).

Table 5.2: Selected chemical drug transporter inhibitors for second screening to investigate SLC22A3 (OCT3) as a potential clozapine transporter

Compound	Concentration	Affected drug transporters	References
Abacavir	10 µM	OCT1, OCT2, OCT3	(Minuesa *et al.* 2009)
Phenytoin	10 µM	OCT3	(Ahlin *et al.* 2008, Hasannejad *et al.* 2004)
Corticosterone	10 µM	OCT3	(Hayer-Zillgen *et al.* 2002)
Corticosterone	100 µM	OCT1, OCT2, OCT3	(Ahlin *et al.* 2008, Hayer-Zillgen *et al.* 2002)

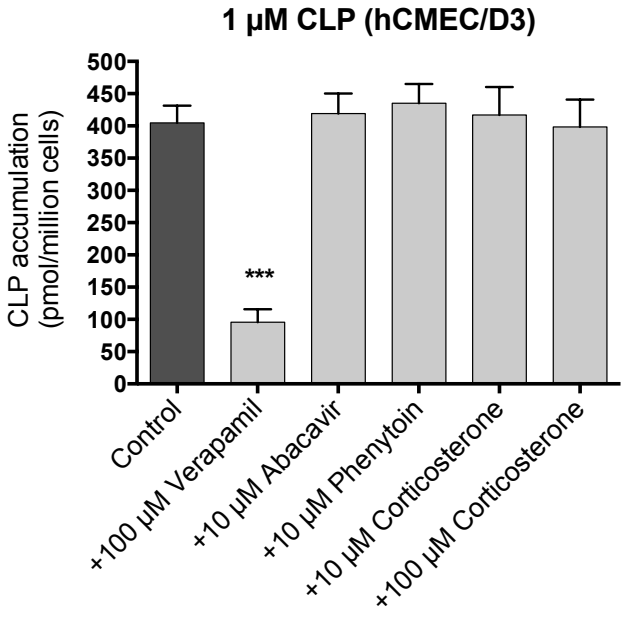


Figure 5.4: Second chemical inhibitor screening to investigate SLC22A3 (OCT3) as a potential clozapine transporter

Accumulation of clozapine (CLP) in the hCMEC/D3 cell line in the presence of different drug transporter inhibitors after 30 minutes at 37 °C. Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with *** for p<0.001

5.3.6. Investigation of potential clozapine transport by SLC22A4 (OCTN1) and SLC22A5 (OCTN2)

The remaining, well-characterised OCT transporter candidates from within the SLC22A family are SLC22A4 (OCTN1) and SLC22A5 (OCTN2). A KCL22 cell line transfected with empty vector or *SLC22A4* was available and CLP investigated as a potential substrate for this particular transporter after 5 and 30 minutes at 37 °C, respectively. The model substrate TEA^+ was utilised as a positive control (Dickens *et al.* 2013a). While no difference in CLP uptake could be observed between control and *SLC22A4*-transfected cells (Figure 5.5 A), TEA^+ accumulation was significantly increased in *SLC22A4*-transfected cells at both time-points, showing functionality of the assay (Figure 5.5 B). These results suggest that SLC22A4 does not mediate CLP uptake into hCMEC/D3 cells.

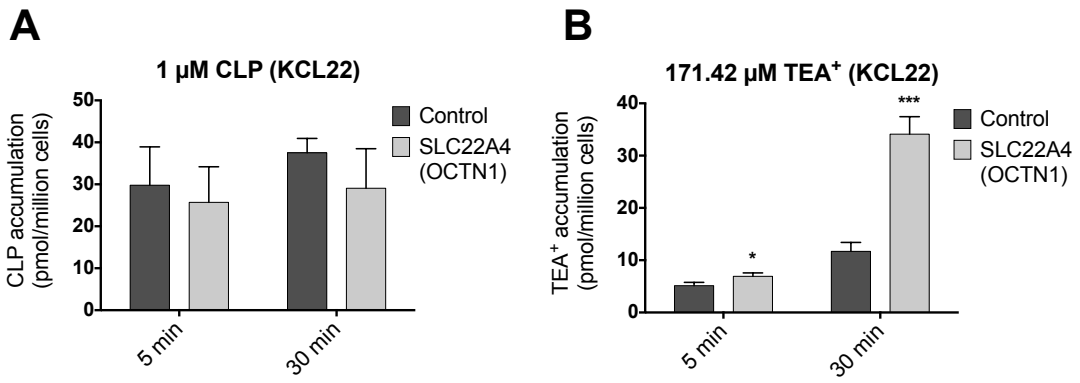


Figure 5.5: Clozapine uptake into *SLC22A4*-transfected KCL22 cells

Clozapine (A) and TEA^+ (B) uptake into empty vector-transfected and *SLC22A4*-transfected KCL22 cells was determined after 5 and 30 minutes at 37 °C, respectively. All data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with * for $p<0.05$ and *** for $p<0.001$. CLP = clozapine, TEA^+ = tetraethylammonium

A transfected cell line was not available to study CLP uptake by SLC22A5. However, gene silencing by means of siRNA transfection had been optimised for the hCMEC/D3 cell line in chapter 3 and was thus used to investigate the impact of *SLC22A5* gene expression on CLP accumulation. In addition, *SLC22A3* was included into the analysis although the results presented in Figure 5.4 did not support the involvement of SLC22A3 (OCT3). The optimal siRNA transfection concentrations had to be determined first. Surprisingly and in contrast to previous studies (Carl *et al.* 2010, Dickens *et al.* 2012), *SLC22A3* mRNA was not detectable using 100 ng cDNA as a template (Figure 5.6 A). Therefore, SLC22A3 can be excluded as the underlying reason for the observed CLP uptake. A strong gene silencing effect was obtained with all three analysed *SLC22A5*-targeting siRNA concentrations (10 nM, 25 nM, 50 nM) (Figure 5.6 A). For comparison, 25 nM siRNAs were chosen as concentrations for the functional assays and the uptake of CLP determined after 1 and 30 minutes at 37 °C, respectively. No difference was observed at both time points comparing CLP uptake into *SLC22A5*-targeting and negative control siRNA transfected cells (Figure 5.6 B+C). The remaining relative *SLC22A5* gene expression was 10 % on average in functional assays using 25 nM siRNAs for transfection (Figure 5.6 D).

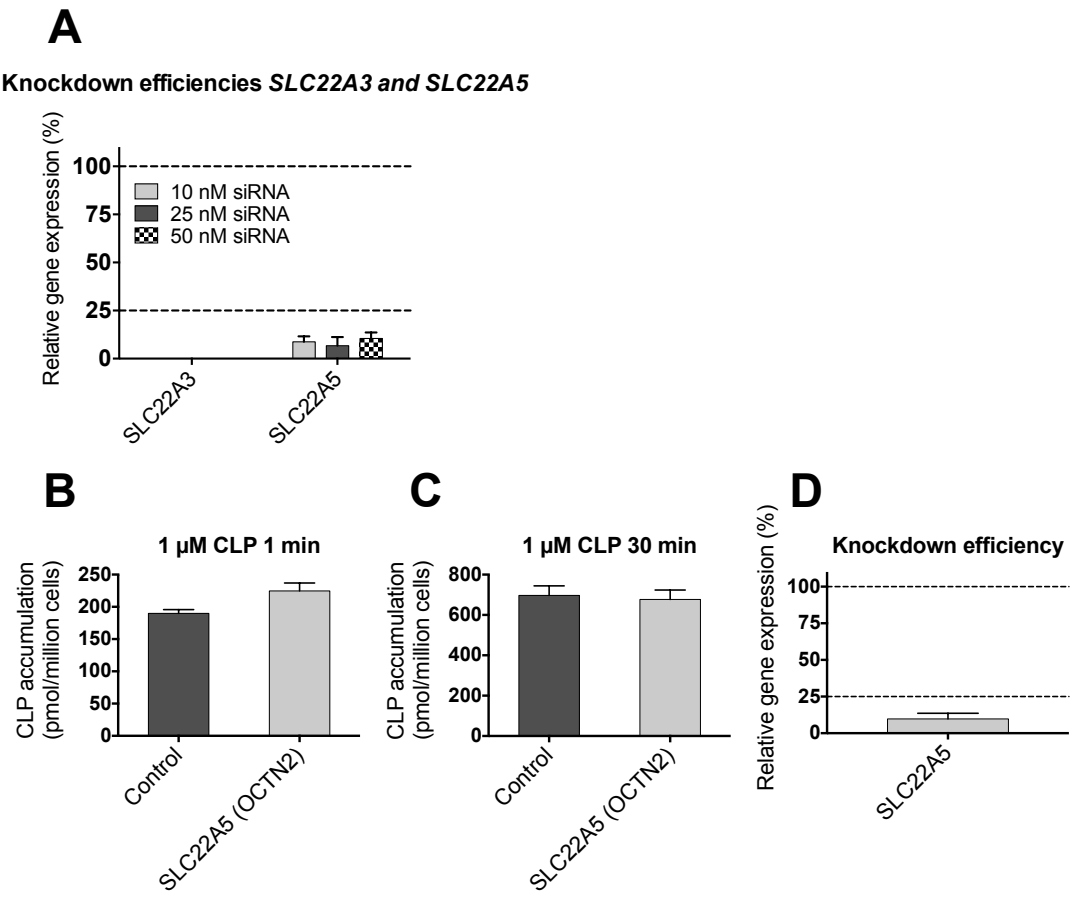


Figure 5.6: Optimisation and functional siRNA screening of a potential clozapine uptake by *SLC22A3* and *SLC22A5*

A) Optimisation of siRNA concentrations (10 nM, 25 nM, 50 nM) targeting *SLC22A3* and *SLC22A5* under optimised delivery conditions. The knockdown efficiency is plotted as the remaining relative gene expression in % as compared to negative control siRNA transfected cells. No gene expression was detectable for *SLC22A3*. Data are expressed as means ± standard deviation and were derived from 1 experiment analysed in quadruplicate; B) Accumulation of clozapine (CLP) in hCMEC/D3 cells after 1 minute at 37 °C utilising a siRNA screening approach. Cells were transfected either with negative control siRNAs or *SLC22A5* targeting siRNAs; C) Accumulation of CLP in hCMEC/D3 cells after 30 minutes at 37 °C utilising a siRNA screening approach. Cells were transfected either with negative control siRNAs or *SLC22A5* targeting siRNAs; D) Relative remaining gene expression in functional experiments as compared to negative control in %; Data in B, C, D are expressed as means ± standard deviation (n= 4 independent experiments each in triplicate (B+C) or quadruplicate (D)

5.3.7. Selection of additional SLC transporter candidates

CLP has so far been analysed as a potential substrate for all functionally characterised organic cation transporters from the SLC22A family that are expressed in the hCMEC/D3 cell line. The results obtained, however, were all negative and a broader screening approach was considered necessary.

As introduced in section 5.3.5, SLC29A4 (ENT4) is a particularly important transporter within the uptake$_2$ system. Together with SLC22A3 (OCT3), it is recognised as a high-capacity monoamine neurotransmitter transporter with expression being detected in various brain regions (Engel *et al.* 2004). Interestingly, SLC29A4 has also been demonstrated to exhibit similar, but still distinct, substrate and inhibitor characteristics as organic cation transporters from the SLC22 family, particularly SLC22A3 (Duan *et al.* 2010, Engel *et al.* 2005, Engel *et al.* 2004). Moreover, CLP has been found to act as a potent inhibitor of SLC29A4-mediated MPP^+ transport (Haenisch *et al.* 2010). Corticosterone up to a concentration of 200 µM, in contrast, had no inhibitory effect on the uptake of serotonin into stably *SLC29A4*-transfected MDCK cells, fitting with the data obtained for CLZ as described in section 5.3.5 (Engel *et al.* 2004). Therefore, SLC29A4 was considered another promising candidate transporter for the potential uptake of CLP into the hCMEC/D3 cell line.

Due to the fact that SLC22A3 and SLC29A4 both exhibit organic cation transporter characteristics and mediate the translocation of monoamine neurotransmitters, other monoamine transporters were also considered interesting candidates. While SLC22A3 and SLC29A4 are important transporters within the extraneuronal uptake$_2$ system, SLC6A2-4 (NET, DAT, SERT) are the main neuronal monoamine transporters of uptake$_1$. Although uptake$_1$ transporters are mostly recognised as mediators for synaptic neurotransmitter re-uptake, expression has also been shown outside the brain. For example, *SLC6A2* has been detected in capillary endothelial cells of the lung (Eisenhofer 2001), *SLC6A3* in endothelial cells of the pancreas (Eisenhofer 2001), and *SLC6A4* in the plasma membrane of platelets (Talvenheimo *et al.* 1980). In addition, CLP is known to interact with many

monoamine neurotransmitter receptors (Novartis Pharmaceuticals Corporation 2013) and thus there may be a link to potential transport by the corresponding transporters, particularly SLC6A2-4.

SLC29A4 is only one out of four transporters classified within the SLC29A subfamily. In addition, the SLC28A subfamily with three transporters is functionally related to SLC29A and both subfamilies are particularly involved in nucleoside transport. Thus, SLC6A2-4, SLC28A1-3 (CNT1-3), and SLC29A1-4 (ENT1-4) are all considered promising candidates for another functional screening approach using RNAi.

As it is unlikely that all the selected transporters are indeed expressed in the hCMEC/D3 cell line, gene expression was initially determined using highly specific and sensitive TaqMan® gene expression assays with 100 ng cDNA per reaction. *SLC22A2* and *SLC22A3* were included to confirm non-expression without prior treatment with the transfection reagent. Gene expression was normalised to *GAPDH* and plotted as reciprocal ΔC_t with lower $1/\Delta C_t$ indicating lower gene expression (Figure 5.7). Non-expression was confirmed for *SLC22A2* and *SLC22A3*. In addition, *SLC6A2, SLC6A3*, *SLC28A1*, and *SLC28A2* were undetectable. *SLC6A4* was also considered not expressed as the C_t values were above 41. The manufacturer defines a C_t above 38 as a "not detectable amplification signal" (Applied Biosystems by Life Technologies 2010). *SLC28A3* showed borderline expression with an average C_t of 38. Interestingly, all *SLC29A* transporter genes were found to be robustly expressed and were included into the siRNA screening with *SLC28A3*.

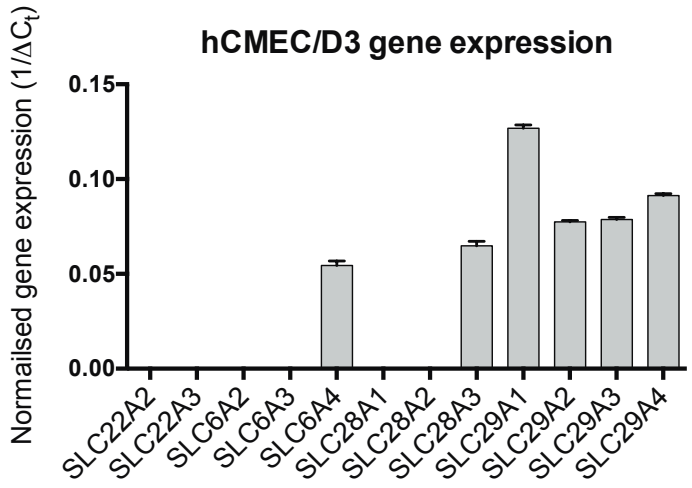


Figure 5.7: Gene expression of selected *SLC* transporter genes in the hCMEC/D3 cell line normalised to *GAPDH*

Gene expression of *SLC22A2-3*, *SLC6A2-4, SLC28A1-3,* and *SLC29A1-4* in the hCMEC/D3 cell line normalised to human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The normalised gene expression is given as the reciprocal delta of both threshold cycles ($1/\Delta C_t$). Empty fields indicate non-detectable gene expression. Data are expressed as means ± standard deviation and were derived from 1 experiment analysed in quadruplicate

5.3.8. siRNA screening

For the delivery of siRNAs into the hCMEC/D3 cell line the best results were obtained by plating 0.1 million cells and transfecting with 0.2 % transfection reagent (see chapter 3 section 3.3.5). siRNA concentrations, however, were optimised on a gene-specific level. Therefore, the knockdown efficiencies for *SLC28A3* and *SLC29A1-4* were determined using 10 nM, 25 nM, and 50 nM siRNAs for transfection, respectively (Figure 5.8). No major differences could be observed and the knockdown efficiency was sufficient for all five genes tested. To keep the assay as consistent as possible, 25 nM siRNAs were chosen for transfection for the functional assays. No accurate comparative gene expression could be determined for *SLC28A3* as the C_t values fluctuated around approximately 39, giving both positive and negative $\Delta\Delta C_t$ values. As discussed before, however, a C_t above 38 was considered to be not detectable (Applied Biosystems by Life Technologies 2011). In addition, even borderline expression can be considered negligible given the effect

size of the observed CLP uptake. Still, the functional screening with *SLC28A3*-targeting siRNAs was included as an additional negative control and the uptake of CLP determined for all five genes after 1 and 30 minutes at 37 °C, respectively (Figure 5.9 A+B). No differences were observed as compared to negative control siRNA transfected cells, indicating that SLC29A1-4 (ENT1-4) do not mediate the observed CLP uptake into hCMEC/D3 cells. The average knockdown efficiencies were adequate for all four genes tested (Figure 5.9 C).

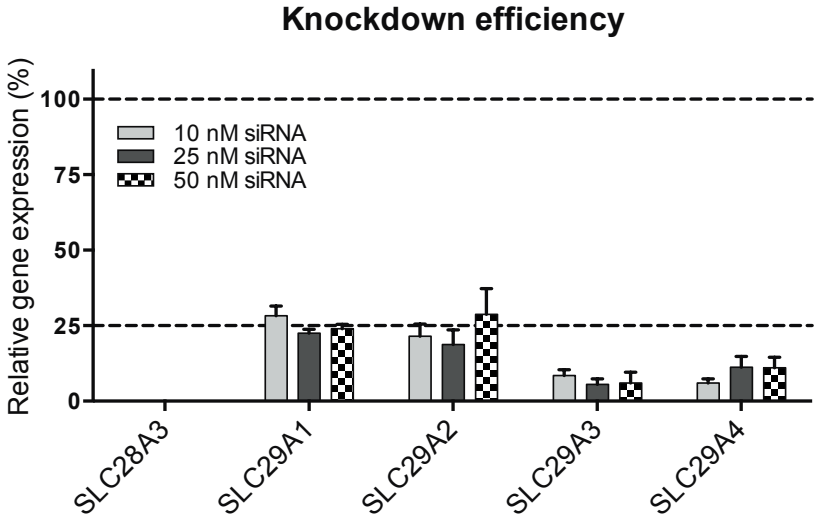


Figure 5.8: Optimisation of siRNA concentrations for gene silencing of selected *SLC* transporters in the hCMEC/D3 cell line

Knockdown efficiencies for *SLC28A3, SLC29A1, SLC29A2, SLC29A3,* and *SLC29A4* genes under optimised delivery conditions using three siRNA concentrations, 10 nM, 25 nM, and 50 nM, respectively. Knockdown efficiency is plotted as the remaining relative gene expression in % as compared to negative control siRNA transfected cells. Analysis of *SLC28A3* failed due to the borderline gene expression. Data are expressed as means ± standard deviation and were derived from 1 experiment analysed in quadruplicate

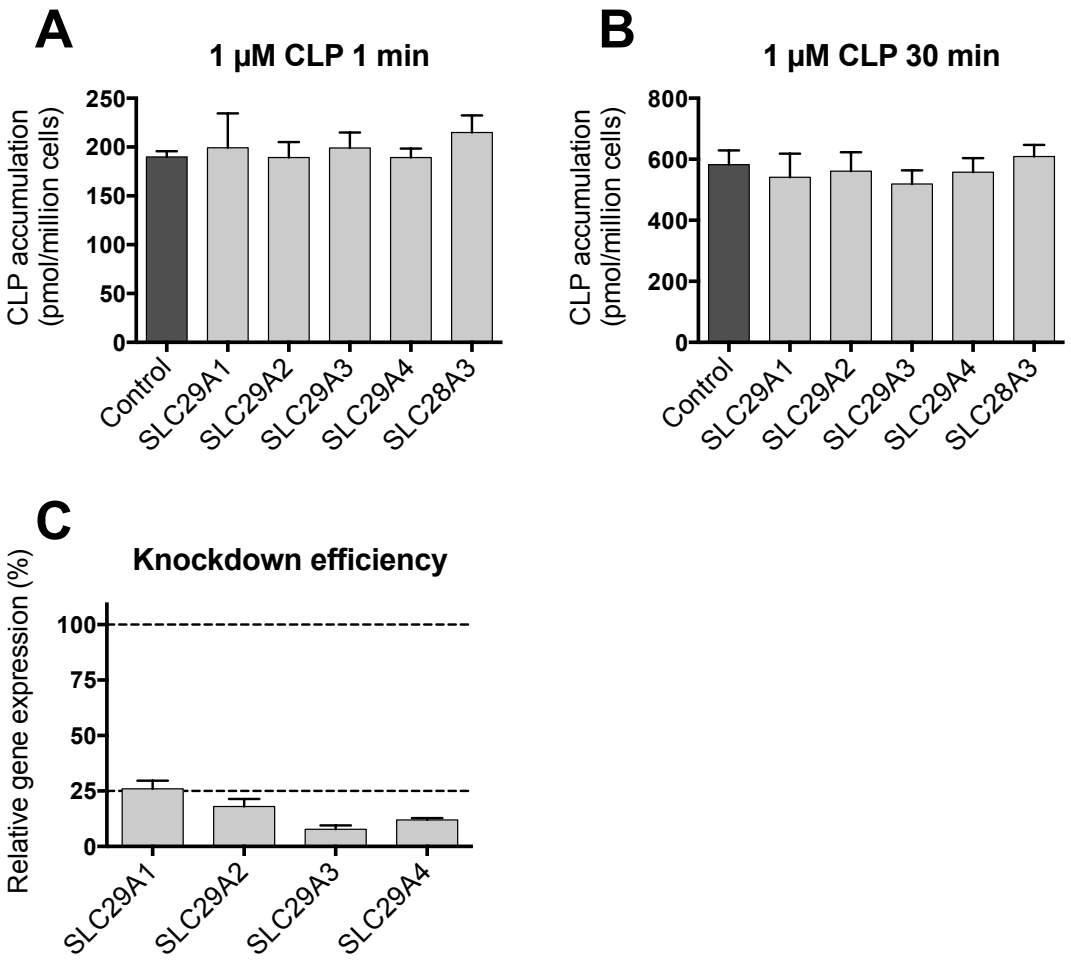


Figure 5.9: Functional siRNA screening of selected SLC transporters in the hCMEC/D3 cell line

A) Accumulation of clozapine (CLP) in hCMEC/D3 cells after 1 minute at 37 °C utilising a siRNA screening approach. Cells were transfected either with negative control siRNAs or corresponding *SLC* transporter targeting siRNAs; B) Accumulation of CLP in hCMEC/D3 cells after 30 minutes at 37 °C utilising a siRNA screening approach. Cells were transfected either with negative control siRNAs or corresponding *SLC* transporter targeting siRNAs; C) Relative remaining gene expression as compared to negative control in %. The analysis of *SLC28A3* failed due to the borderline gene expression. All data are expressed as means ± standard deviation (n= 4 independent experiments each in triplicate (A+B) or quadruplicate (C))

5.3.9. Receptor-internalisation as a potential mechanism for clozapine uptake into the hCMEC/D3 cell line

At this stage it was difficult to identify any promising new drug transporter candidates for analysis. The physicochemical properties of CLP and data from both the literature and this thesis suggest an organic cation transporter as mechanism for CLP uptake. However, all well-characterised organic cation transporters that are expressed in the hCMEC/D3 cell line were tested but with negative outcome. Theoretically, any expressed transporter from within the SLC superfamily could be involved in CLP uptake and the lack of further evidence did not support additional small-scale screening approaches.

Although the observed CLP accumulation appeared to be mediated by an organic cation transporter, other mechanisms such as receptor-internalisation could not be ruled out. CLP interacts with many neurotransmitter receptors, particularly with dopamine and serotonin receptors (Meltzer *et al.* 1989, Novartis Pharmaceuticals Corporation 2013). Most of these are G-protein coupled receptors and CLP-induced receptor-internalisation has been demonstrated for the serotonin receptor 2A ($5\text{-}HT_{2A}$) after stimulation for 15 and/or 30 minutes using 100 nM and 1 µM CLP, respectively (Raote *et al.* 2013, Willins *et al.* 1999). Prazosin was the most potent inhibitor of CLP accumulation in the initial screening assay (5.3.3) and not only is it an OCT inhibitor but it is also an adrenergic α_1-receptor antagonist (Forget *et al.* 2010).

To investigate whether receptor-internalisation was involved, a third chemical inhibitor screening was undertaken. All selected inhibitors, listed in Table 5.3, interact with neurotransmitter receptors and might thus compete with CLP-induced internalisation. The accumulation of 1 µM CLP was determined after 30 minutes at 37 °C in the presence of an excess quantity of inhibitor (20 µM each except for risperidone). Risperidone had to be used at a concentration of 10 µM due to limited solubility. Prazosin and corticosterone were included as positive and

negative controls, respectively, and prazosin concentration adjusted to 20 µM to allow for a better comparison of the potential effect sizes.

Table 5.3: Selected compounds for third inhibitor screening

Compound	Receptors	References
Norepinephrine	Adrenergic (α and ß)	(Kobilka 2011)
Dopamine	Dopamine (D)	(Beaulieu *et al.* 2011)
Serotonin	Serotonin (5-HT)	(Meltzer *et al.* 2003)
Risperidone	Multiple	(Janssen Pharmaceuticals Inc. 2012, Richelson *et al.* 2000)
Quetiapine	Multiple	(AstraZeneca Pharmaceuticals LP 2013, Richelson *et al.* 2000)
Doxazosin	α_1	(Pfizer Inc. 2013)
L-741,626	D_2	(Kulagowski *et al.* 1996)
Propranolol	ß	(Hott *et al.* 2012)
Phentolamine	α	(Hott *et al.* 2012)
M100907	$5\text{-}HT_{2A}$	(Kehne *et al.* 1996)
Sonepiprazole	D_4	(Merchant *et al.* 1996)

A large number of positive results were obtained from this screening approach (Figure 5.10). While the natural monoamine receptor ligands norepinephrine, dopamine, and serotonin did not affect CLP accumulation, all other compounds, with the exception of phentolamine, showed varying degrees of inhibition. Notably, the strongest inhibitory effect was observed with doxazosin, an α_1-receptor blocker like prazosin. Prazosin at 20 µM showed considerably less inhibitory potential (46 % average reduction) compared with 100 µM used for the initial screening (94 % average reduction). Doxazosin was more potent with an average reduction of 81 % when using 20 µM.

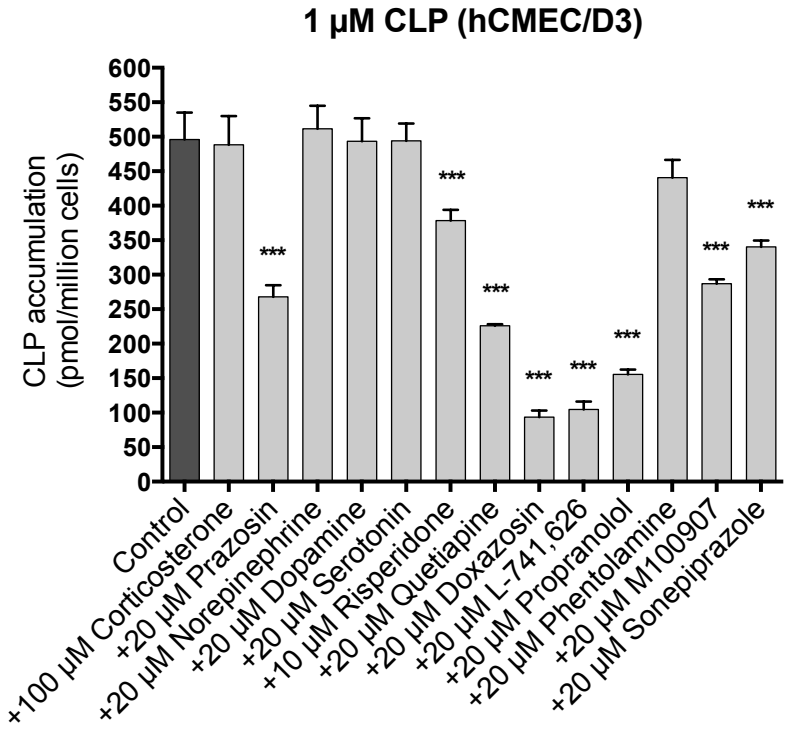


Figure 5.10: Third chemical inhibitor screening to investigate receptor-internalisation as a potential mechanism for clozapine uptake into the hCMEC/D3 cell line

Accumulation of clozapine (CLP) in the hCMEC/D3 cell line after 30 min at 37 °C in the presence of monoamine neurotransmitter receptor agonists/antagonists. Data are expressed as means ± standard deviation (n = 3 independent experiments in triplicate). Significant results are indicated with *** for $p<0.001$

Based on these results, it could be hypothesised that an α-receptor is involved in CLP-internalisation. However, the negative results observed with the α-receptor antagonist phentolamine and the natural receptor ligand norepinephrine do not fit with this hypothesis. Still, it remains possible that other receptor(s) is/are involved in CLP-internalisation. The inhibitor concentrations used were relatively high and additional receptors might be affected. For example, L-741,626, M100907, and sonepiprazole are very selective for receptor subtypes in the nanomolar range (Table 5.3). None of them, however, is receptor-specific at 20 µM (Kehne *et al.* 1996, Kulagowski *et al.* 1996, Merchant *et al.* 1996).

The data suggest that CLP accumulation could indeed be a result from receptor-internalisation. In addition, however, the selected inhibitors may also interact with an unidentified organic cation transporter. A larger functional screening approach, ideally including all expressed SLC transporters and neurotransmitter receptors, would be the most promising further strategy to identify the underlying mechanism for CLP uptake.

5.4. Discussion

Evidence from the literature and chapter 4 suggested that cellular CLP accumulation may be mediated by an unidentified uptake transporter. A screening approach was applied to identify and characterise this process. Indeed, CLP accumulation in the hCMEC/D3 cell line could be inhibited by up to 94 % and the overall inhibitor profile suggested that an organic cation transporter was involved.

The uptake of CLP was analysed in more detail and found to follow classical Michaelis-Menten kinetics with a V_{max} of 3299 pmol/million cells and a K_m of 35.93 µM. Recently, the kinetics for LTG uptake has been described utilising the same cell line. The SLC22A1 (OCT1)-mediated uptake of LTG was found to have a V_{max} of 385 pmol/million cells and a K_m of 62 µM (Dickens *et al.* 2012). A K_m of 35.93 µM for CLP uptake is notably lower than the K_m for SLC22A1-mediated LTG uptake. Classically, this would imply a higher-affinity but lower-capacity transport process for CLP relative to LTG. However, CLP peak plasma levels are around 1 µM (Novartis Pharmaceuticals Corporation 2013) and thus a K_m of 35.93 µM still indicates a high-capacity transporter at clinically important concentrations.

The observed CLP uptake into hCMEC/D3 cells was not corrected for any background transport as has been done for LTG (Dickens *et al.* 2012). However, inhibition up to 94 % indicates that, despite the lipophilic characteristics, CLP accumulation was primarily the result from an active uptake process. Interestingly, a slightly lower but still similar K_m of 18.8 µM has previously been observed for CLP uptake into promyelocytic leukaemia HL-60 cells (Henning *et al.* 2002).

In an attempt to identify the unknown CLP transporter, the mRNA expression levels of *SLC22A2-3, SLC22A5*, *SLC6A2-4*, *SLC28A1-3*, and *SLC29A1-4* were analysed in the hCMEC/D3 cell line, selected based on evidence from the literature and results from the inhibitor screenings (see results section for details). *SLC22A5*, *SLC28A3*, and *SLC29A1-4* were found to be expressed and the transporters functionally analysed using a siRNA screening approach. In addition, a potential transport by SLC22A4 (OCTN1) was analysed using transfected KCL22 cell lines. None of the transporters, however, had an impact on CLP accumulation. SLC22A1, analysed in chapter 4 section 4.3.2, was also negative. Surprisingly and in contrast to previous reports (Carl *et al.* 2010, Dickens *et al.* 2012), *SLC22A3* was not detectable in the hCMEC/D3 cell line. The exact underlying reason for this observation is unclear but chromosomal rearrangements have been reported for the hCMEC/D3 cell line that might explain the loss of *SLC22A3* expression as compared to the cells used by Carl *et al*. and Dickens *et al.* (Mkrtchyan *et al.* 2009).

CLP-mediated receptor-internalisation has previously been demonstrated for the 5-HT_{2A} receptor, providing an alternative hypothetical mechanism for the observed CLP accumulation. Further screening was carried out to analyse the effect of several monoamine receptor agonists/antagonists on CLP accumulation. While most antagonists exhibited varying degrees of inhibition, all natural agonists, namely norepinephrine, dopamine, and serotonin, had no effect on CLP accumulation. It remains unclear if the observed inhibition is a result of an impaired receptor-internalisation, impaired transport by an unknown organic cation transporter, or both.

The data does not support additional small-scale screening approaches. Instead, more extensive screening will be necessary, ideally including all expressed SLC transporters and monoamine receptors. This screening would be approached either clinically or functionally. Functionally, a siRNA library screening could be a promising strategy as discussed for the unknown TPM efflux transporter (see chapter 3 section 3.4). However, this is an expensive and labour-intensive approach. In addition, siRNA screenings, including the ones discussed above, have some limitations. First, siRNA transfections can substantially lower the expression of the

targeted transporter gene but the remaining expressed protein might still have enough capacity to overcome this reduction. Second, functional redundancy could mask impaired accumulation if the expression of only one transporter is reduced. Third, and as seen in chapter 3 with *ABCC5,* it is likely that some transporters will be difficult to target with siRNAs.

Clinically, a GWAS with samples from a CLP-treated patient cohort may be a promising strategy to identify variants of the unknown transporter that could be associated with study outcomes such as treatment response or the occurrence of adverse events. This transporter could then be further analysed and characterised functionally. Such an approach has successfully been applied before. For example, variants of *SLCO1B1* have been associated with statin-induced myopathy and variants of *ABCG2* with elevated urate levels (Dehghan *et al.* 2008, Kottgen *et al.* 2013, Link *et al.* 2008). However, there are some limitations to this approach as well. Most importantly, a clinical study has to be undertaken first and DNA samples from all patients collected. In addition, a critical number of patients will be required to achieve enough power for statistical analysis. Finally, variants of the unknown CLP transporter have to be associated with the study outcome(s) to be detectable.

In conclusion, a potent CLP uptake process has been identified and characterised in the hCMEC/D3 cell line as an *in vitro* model of the BBB. This may critically affect CLP treatment efficacy in the brain. In addition, these results support previous studies, which showed that a CLP transporter may also be involved in severe side effects such as agranulocytosis (Bergemann *et al.* 2007, Henning *et al.* 2002). A large-scale functional or clinical screening will be necessary to identify the exact transport mechanism.

5.5. References

Ahlin, G.*, et al.* (2008) Structural requirements for drug inhibition of the liver specific human organic cation transport protein 1. *J Med Chem* **51**, 5932-5942

Allen, J. D.*, et al.* (2002) Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Molecular cancer therapeutics* **1**, 417-425

Applied Biosystems by Life Technologies (2011) Real-time PCR: Understanding Ct http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_053906.pdf

Applied Biosystems by Life Technologies (2010) White Paper TaqMan(R) Assays QPCR Guarantee Program http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_088754.pdf

AstraZeneca Pharmaceuticals LP (2013) Seroquel Prescribing Information

Badagnani, I.*, et al.* (2006) Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J Pharmacol Exp Ther* **318**, 521-529

Beaulieu, J. M., and Gainetdinov, R. R. (2011) The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacological reviews* **63**, 182-217

Bergemann, N.*, et al.* (2007) High clozapine concentrations in leukocytes in a patient who developed leukocytopenia. *Progress in neuro-psychopharmacology & biological psychiatry* **31**, 1068-1071

Boesch, D.*, et al.* (1991) In vivo circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res* **51**, 4226-4233

Burckhardt, G., and Burckhardt, B. C. (2011) In vitro and in vivo evidence of the importance of organic anion transporters (OATs) in drug therapy. *Handb.Exp.Pharmacol.*, 29-104

Carl, S. M.*, et al.* (2010) ABC and SLC transporter expression and pot substrate characterization across the human CMEC/D3 blood-brain barrier cell line. *Mol.Pharm.* **7**, 1057-1068

Chen, Z. S.*, et al.* (2003) Characterization of the transport properties of human multidrug resistance protein 7 (MRP7, ABCC10). *Molecular pharmacology* **63**, 351-358

Dehghan, A.*, et al.* (2008) Association of three genetic loci with uric acid concentration and risk of gout: a genome-wide association study. *Lancet* **372**, 1953-1961

Dickens, D.*, et al.* (2012) Lamotrigine is a substrate for OCT1 in brain endothelial cells. *Biochemical pharmacology* **83**, 805-814

Dickens, D.*, et al.* (2013a) Transport of gabapentin by LAT1 (SLC7A5). *Biochemical pharmacology* **85**, 1672-1683

Dickens, D.*, et al.* (2013b) A multi-system approach assessing the interaction of anticonvulsants with P-gp. *PloS one* **8**, e64854

Doran, A.*, et al.* (2005) The impact of P-glycoprotein on the disposition of drugs targeted for indications of the central nervous system: evaluation using the MDR1A/1B knockout mouse model. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 165-174

Duan, H., and Wang, J. (2010) Selective transport of monoamine neurotransmitters by human plasma membrane monoamine transporter and organic cation transporter 3. *J Pharmacol Exp Ther* **335**, 743-753

Eiermann, B.*, et al.* (1997) The involvement of CYP1A2 and CYP3A4 in the metabolism of clozapine. *Br J Clin Pharmacol* **44**, 439-446

Eisenhofer, G. (2001) The role of neuronal and extraneuronal plasma membrane transporters in the inactivation of peripheral catecholamines. *Pharmacology & therapeutics* **91**, 35-62

Engel, K., and Wang, J. (2005) Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Molecular pharmacology* **68**, 1397-1407

Engel, K., Zhou, M., and Wang, J. (2004) Identification and characterization of a novel monoamine transporter in the human brain. *The Journal of biological chemistry* **279**, 50042-50049

Forget, B.*, et al.* (2010) Noradrenergic alpha1 receptors as a novel target for the treatment of nicotine addiction. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **35**, 1751-1760

Giacomini, K. M.*, et al.* (2010) Membrane transporters in drug development. *Nat.Rev.Drug Discov.* **9**, 215-236

Haenisch, B., and Bonisch, H. (2010) Interaction of the human plasma membrane monoamine transporter (hPMAT) with antidepressants and antipsychotics. *Naunyn-Schmiedeberg's archives of pharmacology* **381**, 33-39

Haenisch, B.*, et al.* (2012) Interaction of antidepressant and antipsychotic drugs with the human organic cation transporters hOCT1, hOCT2 and hOCT3. *Naunyn-Schmiedeberg's archives of pharmacology* **385**, 1017-1023

Hartter, S.*, et al.* (2003) How does the benzamide antipsychotic amisulpride get into the brain?--An in vitro approach comparing amisulpride with clozapine. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **28**, 1916-1922

Hasannejad, H.*, et al.* (2004) Human organic cation transporter 3 mediates the transport of antiarrhythmic drugs. *European journal of pharmacology* **499**, 45-51

Hayer-Zillgen, M., Bruss, M., and Bonisch, H. (2002) Expression and pharmacological profile of the human organic cation transporters hOCT1, hOCT2 and hOCT3. *British journal of pharmacology* **136**, 829-836

Henning, U.*, et al.* (2002) Uptake of clozapine into HL-60 promyelocytic leukaemia cells. *Pharmacopsychiatry* **35**, 90-95

Hott, S. C.*, et al.* (2012) Both alpha1- and beta1-adrenoceptors in the bed nucleus of the stria terminalis are involved in the expression of conditioned contextual fear. *British journal of pharmacology* **167**, 207-221

Janssen Pharmaceuticals Inc. (2012) Risperdal Prescribing Information

Kane, J.*, et al.* (1988) Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. *Archives of general psychiatry* **45**, 789-796

Kannan, P.*, et al.* (2011) The "specific" P-glycoprotein inhibitor Tariquidar is also a substrate and an inhibitor for breast cancer resistance protein (BCRP/ABCG2). *ACS chemical neuroscience* **2**, 82-89

Kapur, S.*, et al.* (2006) How antipsychotics work-from receptors to reality. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* **3**, 10-21

Kehne, J. H.*, et al.* (1996) Preclinical characterization of the potential of the putative atypical antipsychotic MDL 100,907 as a potent 5-HT2A antagonist with a favorable CNS safety profile. *J Pharmacol Exp Ther* **277**, 968-981

Keppler, D. (2011) Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handbook of experimental pharmacology*, 299-323

Khamdang, S.*, et al.* (2002) Interactions of human organic anion transporters and human organic cation transporters with nonsteroidal anti-inflammatory drugs. *J Pharmacol Exp Ther* **303**, 534-539

Kobilka, B. K. (2011) Structural insights into adrenergic receptor function and pharmacology. *Trends in pharmacological sciences* **32**, 213-218

Kottgen, A.*, et al.* (2013) Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. *Nature genetics* **45**, 145-154

Kulagowski, J. J.*, et al.* (1996) 3-((4-(4-Chlorophenyl)piperazin-1-yl)-methyl)-1H-pyrrolo-2,3-b-pyridine: an antagonist with high affinity and selectivity for the human dopamine D4 receptor. *J Med Chem* **39**, 1941-1942

Letschert, K.*, et al.* (2005) Vectorial transport of the peptide CCK-8 by double-transfected MDCKII cells stably expressing the organic anion transporter OATP1B3 (OATP8) and the export pump ABCC2. *J Pharmacol Exp Ther* **313**, 549-556

Link, E.*, et al.* (2008) SLCO1B1 variants and statin-induced myopathy--a genomewide study. *The New England journal of medicine* **359**, 789-799

Matsson, P.*, et al.* (2007) A global drug inhibition pattern for the human ATP-binding cassette transporter breast cancer resistance protein (ABCG2). *J.Pharmacol.Exp.Ther.* **323**, 19-30

Matsson, P.*, et al.* (2009) Identification of novel specific and general inhibitors of the three major human ATP-binding cassette transporters P-gp, BCRP and MRP2 among registered drugs. *Pharm.Res.* **26**, 1816-1831

Meltzer, H. Y.*, et al.* (2003) Serotonin receptors: their key role in drugs to treat schizophrenia. *Progress in neuro-psychopharmacology & biological psychiatry* **27**, 1159-1172

Meltzer, H. Y., Matsubara, S., and Lee, J. C. (1989) Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin2 pKi values. *J Pharmacol Exp Ther* **251**, 238-246

Merchant, K. M.*, et al.* (1996) Pharmacological characterization of U-101387, a dopamine D4 receptor selective antagonist. *J Pharmacol Exp Ther* **279**, 1392-1403

Minuesa, G.*, et al.* (2009) Transport of lamivudine [(-)-beta-L-2',3'-dideoxy-3'-thiacytidine] and high-affinity interaction of nucleoside reverse transcriptase inhibitors with human organic cation transporters 1, 2, and 3. *J Pharmacol Exp Ther* **329**, 252-261

Mistry, P.*, et al.* (2001) In vitro and in vivo reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. *Cancer Res* **61**, 749-758

Miyamoto, S.*, et al.* (2012) Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. *Molecular psychiatry* **17**, 1206-1227

Mkrtchyan, H.*, et al.* (2009) Molecular cytogenetic characterization of the human cerebral microvessel endothelial cell line hCMEC/D3. *Cytogenetic and genome research* **126**, 313-317

National Institute for Health and Clinical Excellence (2009) Schizophrenia: core interventions in the treatment and management of schizophrenia in adults in primary and secondary care

Nies, A. T.*, et al.* (2011) Organic Cation Transporters (OCTs, MATEs), In Vitro and In Vivo Evidence for the Importance in Drug Therapy. *Handb.Exp.Pharmacol.* **201**, 105-167

Novartis Pharmaceuticals Corporation (2013) Clozaril Prescribing Information

Ohashi, R.*, et al.* (1999) Na(+)-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. *J Pharmacol Exp Ther* **291**, 778-784

Pfizer Inc. (2013) Cardura Labelling Information

Pirmohamed, M.*, et al.* (1995) Metabolism and bioactivation of clozapine by human liver in vitro. *J Pharmacol Exp Ther* **272**, 984-990

Raote, I., Bhattacharyya, S., and Panicker, M. M. (2013) Functional selectivity in serotonin receptor 2A (5-HT2A) endocytosis, recycling, and phosphorylation. *Molecular pharmacology* **83**, 42-50

Reid, G.*, et al.* (2003) The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc.Natl.Acad.Sci.U.S.A* **100**, 9244-9249

Richelson, E., and Souder, T. (2000) Binding of antipsychotic drugs to human brain receptors focus on newer generation compounds. *Life sciences* **68**, 29-39

Schinkel, A. H.*, et al.* (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J.Clin.Invest* **97**, 2517-2524

Schmitt, U.*, et al.* (2012) In vitro P-glycoprotein efflux inhibition by atypical antipsychotics is in vivo nicely reflected by pharmacodynamic but less by pharmacokinetic changes. *Pharmacology, biochemistry, and behavior* **102**, 312-320

Sugano, K.*, et al.* (2010) Coexistence of passive and carrier-mediated processes in drug transport. *Nature reviews. Drug discovery* **9**, 597-614

Sun, Y. L.*, et al.* (2013) Reversal of MRP7 (ABCC10)-mediated multidrug resistance by tariquidar. *PloS one* **8**, e55576

Talvenheimo, J., and Rudnick, G. (1980) Solubilization of the platelet plasma membrane serotonin transporter in an active form. *The Journal of biological chemistry* **255**, 8606-8611

van de Waterbeemd, H.*, et al.* (1998) Estimation of blood-brain barrier crossing of drugs using molecular size and shape, and H-bonding descriptors. *Journal of drug targeting* **6**, 151-165

van Os, J., and Kapur, S. (2009) Schizophrenia. *Lancet* **374**, 635-645

Wang, J. S.*, et al.* (2006) Evaluation of antipsychotic drugs as inhibitors of multidrug resistance transporter P-glycoprotein. *Psychopharmacology* **187**, 415-423

Wang, J. S.*, et al.* (2008) Antipsychotic drugs inhibit the function of breast cancer resistance protein. *Basic & clinical pharmacology & toxicology* **103**, 336-341

Willins, D. L.*, et al.* (1999) Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors in vitro and in vivo. *Neuroscience* **91**, 599-606

World Health Organisation (2014) Schizophrenia http://www.who.int/mental_health/management/schizophrenia/en/

Yabuuchi, H.*, et al.* (1999) Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations. *J Pharmacol Exp Ther* **289**, 768-773

Chapter 6

Final discussion

Contents

6.1. The emerging role of drug transporters in pharmacokinetics

A large number of licensed drugs, particularly hydrophobic drugs, have to undergo different biotransformation steps to be eliminated from the body. The enzymes involved in drug metabolism are thus important components to be considered in drug pharmacokinetics and have been studied for decades. In the middle of the 20th century, R.T. Williams first proposed that xenobiotic biotransformation can essentially be classified into phase I and phase II reactions (Williams 1959). Phase I reactions were described as activation or inactivation steps through oxidation, reduction, hydrolysis, or a combination of these. Phase II was referred to as the synthesis step that is particularly characterised by conjugation reactions such as glucuronidation, acetylation, or glutathione conjugation (Bachmann 2009, David Josephy *et al.* 2005, Williams 1959). Later, this classification was extended with the introduction of the phase III and phase 0 systems to account for the uptake and efflux of drugs and conjugates across plasma membranes (Doring *et al.* 2014). Phase 0 refers to the uptake of drugs by members of the SLC superfamily of drug transporters, while phase III refers to efflux by transporters from the ABC family (Doring *et al.* 2014, Ishikawa 1992, Petzinger *et al.* 2006).

David Josephy *et al*. critically noted in 2005 that the terms phase I, phase II, and phase III are misleading as they imply a sequential order that is not always true (David Josephy *et al.* 2005). In fact, drugs may only undergo phase II metabolism without prior phase I (e.g. LTG, see chapter 1 section 1.3.3.2) and drug efflux is not restricted to conjugates and the final elimination step (David Josephy *et al.* 2005). Instead, the authors suggested classifying drug metabolism more systematically according to the chemical reactions that occur, namely oxidation, reduction, hydrolysis, and nucleophilic trapping reactions (David Josephy *et al.* 2005). However, the classification of chemical reactions and transport processes into phases is still commonly used in the field of pharmacology.

An estimated three-fourths of all licensed drugs are subject to metabolism by mixed-function oxidases from the CYP family (Bachmann 2009). CYP enzymes have now been studied for decades and are recognised as major determinants of a drug´s pharmacokinetic properties. In addition, they are known to be of particular importance as mediators for pharmacokinetic drug-drug interactions if two co-administered drugs are metabolised by the same CYP enzyme(s) (Bachmann 2009). For example, CYP3A4/5 metabolise about 50 % of all clinically used drugs and thus the risk for drug-drug interactions is high (Bachmann 2009).

The metabolism by important CYP enzymes is now routinely evaluated in the development of investigational new drugs (INDs). In their 2012 draft guideline, the FDA recommends to begin with the *in vitro* analysis of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Depending on the outcome, the analysis can then be extended to *in vivo* investigations and mechanistic modelling (United States Food and Drug Administration 2012).

In contrast to CYP enzymes, drug transporters have not received much attention in drug development and recommendations were not available (except for ABCB1) until 2012 (Giacomini *et al.* 2010, United States Food and Drug Administration 2012). Based on the white paper published by the ITC in 2010, the FDA included a total of seven transporters into their latest (2012) draft recommendations for the evaluation of potential drug-drug interactions in IND development, namely ABCB1 (MDR1, Pgp), ABCG2 (BCRP), SLC22A2 (OCT2), SLC22A6 (OAT1), SLC22A8 (OAT3), SLCO1B1 (OATP1B1), and SLCO1B3 (OATP1B3) (Figure 6.1). Recently, the ITC published a list of additional transporters (e.g. ABCC2 (MRP2)) highlighting the discussions of the second ITC workshop held in 2012 (Giacomini *et al.* 2013). The publication of a series of additional white paper recommendations was announced and it is likely that the FDA will follow these recommendations in future guidelines. Overall, drug transporters are now being recognised as important components in pharmacokinetics and in pharmacokinetic drug-drug interactions. As compared to CYP enzymes, however, drug transporters are just beginning to be investigated more systematically and the large number of

uncharacterised human SLC and ABC transporters make this an exciting and growing field of research.

6.2. *In vitro* transporter assays

Drug transport across the BBB is of fundamental importance for the efficacy of CNS targeting drugs. In addition, the restriction or uptake of drugs across the BBB may determine the side effect profile of many non-CNS targeting drugs. While INDs are now increasingly characterised for their interaction with selected drug transporters (United States Food and Drug Administration 2012), data for currently marketed drugs are very limited or absent. About 30 % of all epilepsy patients do not achieve adequate seizure control with currently licensed AEDs (Brodie *et al.* 2010, World Health Organisation 2009). In schizophrenia, CLP is a last-resort treatment option for otherwise refractory patients. However, about 70 % of these initially refractory patients also fail to achieve adequate control over their symptoms with CLP (Chakos *et al.* 2001, Kane *et al.* 1988, Lieberman *et al.* 1994).

AEDs and APDs both target the CNS and thus have to cross the BBB in order to become effective. Uptake and/or efflux transporters may be critical components for the effectiveness of both classes of neurological treatments and thus provide a pathophysiological basis explaining treatment failure in this large proportion of patients. In addition, membrane transporters may be critical components in the occurrence of side effects such as CLP-induced agranulocytosis.

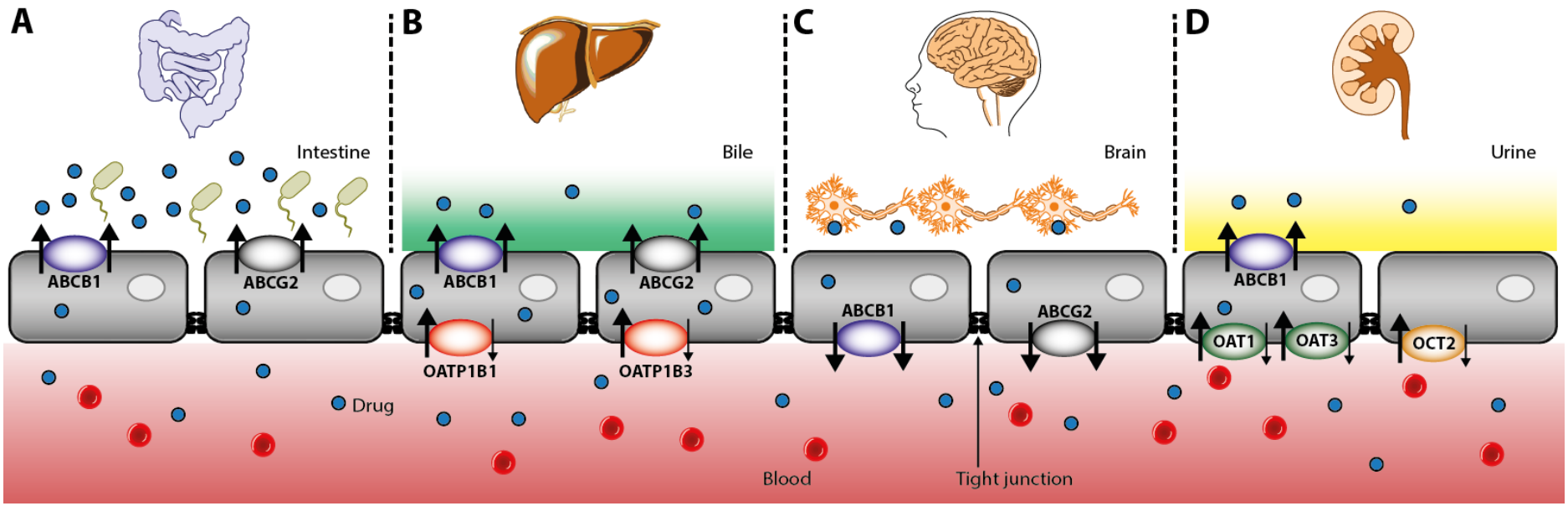


Figure 6.1: Membrane transporters currently recommended by the United States Food and Drug Administration (FDA) for the evaluation of potential drug-drug interactions in drug development

Simplified model illustrating the subcellular localisation of seven important membrane transporters at four pharmacologically important barriers, epithelial cells of the intestine (A), liver hepatocytes (B), brain endothelial cells (C), and tubule epithelial kidney cells (D); Currently recommended efflux transporters include ABCB1 (MDR1, Pgp) and ABCG2 (BCRP) while currently recommended uptake transporters include SLCO1B1 (OATP1B1), SLCO1B3 (OATP1B3), SLC22A2 (OCT2), SLC22A6 (OAT1), and SLC22A8 (OAT3). Model based on Giacomini *et al*., 2010

In chapters 3 and 5 of this thesis, the hCMEC/D3 cell line (Weksler *et al.* 2005) was applied as an *in vitro* model of the BBB to investigate the effect of various chemical drug transporter inhibitors on the accumulation of six AEDs and the APD CLP. Increased accumulation of TPM was observed in the presence of MK571 and montelukast, suggesting an unidentified efflux transporter being involved in TPM accumulation. The accumulation of CLP was reduced in the presence of the typical organic cation transporter inhibitors prazosin and verapamil. In addition, many neurotransmitter receptor-targeting drugs significantly reduced the accumulation of CLP. These data suggest an unidentified uptake transporter and/or receptor-internalisation being involved in CLP accumulation. Subsequent investigations by means of siRNA transfections or stably transfected cell lines, however, could not identify the exact transport processes involved in TPM and CLP accumulation, although the assays were shown to be functional.

Due to the fact that the human genome encodes for about 390 SLC and 48 ABC transporters (excluding pseudogenes), the overall fraction of tested transporters is very small. The chemical inhibitors utilised cannot be considered as absolutely specific and thus the impact of the majority of SLC and ABC transporters on TPM and CLP accumulation remains unknown. Therefore and in addition to potential issues with functional redundancy (see discussion sections 3.4 and 5.4 in chapters 3 and 5), the sought-after transporters or receptors have likely been missed. Due to the lack of further evidence from the literature, a broader screening approach, ideally including all expressed SLC and ABC transporters as well as neurotransmitter receptors, is necessary.

Functional siRNA library screenings could be a strategy to overcome the limited number of transporters tested, but still carries the risks of functional redundancy and insufficient knockdown efficiencies. A widely applied method for the *in vitro* identification and characterisation of drug transporters is by means of transporter assays utilising recombinant cell lines (Giacomini *et al.* 2010). The value of this method was demonstrated in chapters 2 and 4 to characterise CBZ as a potential substrate of ABCC2 (MRP2) and to investigate SLC22A1 (OCT1)-mediated drug-drug interactions with LTG. The same method, however, cannot readily be

applied for a broader screening approach as would be required to follow up on the results in chapters 3 and 5 with TPM and CLP, respectively. The large quantity of encoded human SLC and ABC transporters and the simultaneous absence of transfected cell lines for most of these transporters limits the applicability.

Currently, there are no reliable methods described that would allow for large-scale functional screenings. Lanthaler *et al*. recently reported the successful implementation of a homozygous, diploid gene-deletion collection of *Saccharomyces cerevisiae* mutants to carry out cytotoxicity screenings as a measure for unidentified drug transport processes (Giaever *et al.* 2002, Lanthaler *et al.* 2011). Although this is an elegant method allowing high-throughput screenings, the indirect read-out by means of cytotoxicity, the risk of functional redundancy, and species-differences, also limit the applicability of this approach.

While the hCMEC/D3 cell line proved to be valuable for the identification of TPM efflux and CLP uptake in the screening experiments, there are critical limitations and drawbacks to this cell line being used in more systematic BBB screenings, for example in pre-clinical development. First, the absence and thus missing interaction with other structures from the neurovascular unit may alter the BBB characteristics of this isolated endothelial cell line, particularly with regards to barrier properties (Lippmann *et al.* 2012, Urich *et al.* 2012). The transepithelial resistance (TEER) of confluent hCMEC/D3 cell monolayers is relatively low and paracellular diffusion higher when compared to other reference BBB *in vitro* models (bovine and porcine cerebral microvessel endothelial cells) for compounds < 4,000 Da (Lippmann *et al.* 2012, Poller *et al.* 2008, Urich *et al.* 2012, Weksler *et al.* 2013, Weksler *et al.* 2005). Most drugs have a molecular weight far below 4,000 Da, e.g. TPM is 339 Da and CLP is 327 Da, and thus bi-directional transporter assays are not suitable to investigate drug transport across a hCMEC/D3 cell monolayer.

Recently, Ulrich *et al*. compared the global gene expression profile from cultured hCMEC/D3 cells, cultured primary human brain endothelial cells (cphBECs), and freshly isolated mouse brain endothelial cells (fmBECs) on the mRNA level (Urich *et al.* 2012). While hCMEC/D3 and cphBECS exhibited very similar overall

gene expression profiles, there were marked differences with fmBECs. In agreement with the observed low TEER and poor paracellular restriction, substantially reduced mRNA levels encoding for the important tight junction proteins claudin 5, occludin, and JAM 2 were noted in hCMEC/D3 and cphBEC cells when compared with fmBECs (Urich *et al.* 2012). In addition, strongly reduced mRNA levels encoding for different SLC and ABC transporters such as ABCB1 (MDR1, Pgp), ABCG2 (BCRP), SLC7A5 (LAT1), and SLC2A1 (GLUT1) have been reported although the functional effect sizes remain unknown and functionality of SLC7A5 has recently been demonstrated for the hCMEC/D3 cell line (Dickens *et al.* 2013, Urich *et al.* 2012). The authors concluded that primary or immortalised human brain endothelial cells both lose some of their BBB specific features and exhibit an endothelial cell phenotype that is more generic (Urich *et al.* 2012). These cross-species comparisons, however, have to be taken with some care. For example, another study reported substantially lower *ABCG2* mRNA levels in isolated human brain microvascular endothelial cells (hBMECs) as compared to isolated BMECs derived from mouse, rat, cow, and pig (Warren *et al.* 2009).

A second limitation of the hCMEC/D3 cell line is that it was isolated from resected brain tissue derived from an epilepsy patient and the expression profile of drug transporters may thus be altered as compared to healthy individuals (Weksler *et al.* 2005). Third and most critical for comparing data between different studies using the hCMEC/D3 cell line is that genetic shifts have been reported including a complex karyotype and chromosomal rearrangements (Mkrtchyan *et al.* 2009). Evidence for this observation was obtained in chapter 5 where *SLC22A3* mRNA, in contrast to previous studies (Carl *et al.* 2010, Dickens *et al.* 2012), was not detectable using a large quantity (100 ng) of cDNA template.

Taken together, the hCMEC/D3 cell line is a suitable model cell line for initial screening of drug transport into the human brain as has been performed in chapters 3 and 5. For systematic screening in drug development, however, the limitations discussed encourage the development of more robust models with improved barrier properties to better mimic the situation *in vivo*.

Recently, an interesting new method has been published that describes the generation of brain hBMECs derived from human pluripotent stem cells (hPSCs) (Lippmann *et al.* 2012). Co-differentiation with other neural cells, initially with unconditioned medium followed by incubation in endothelial cell medium, yielded high amounts of hBMECs that were then successfully isolated and subcultured to obtain pure hBMEC monolayers (Lippmann *et al.* 2012). The hBMEC monolayers have been characterised and were found to exhibit many BBB characteristics, particularly the presence of well-organised tight junctions and the expression of SLC and ABC transporters such as *SLC2A1*, *SLC7A5*, *ABCB1*, *ABCG2*, *ABCC1*, *ABCC2*, *ABCC4*, and *ABCC5* (Lippmann *et al.* 2012). Most notably, TEER values were substantially higher than has been reported for the hCMEC/D3 cell line (Forster *et al.* 2008, Hatherell *et al.* 2011, Weksler *et al.* 2005) and co-culture with astrocytes further increased TEER values to a level similar to that observed *in vivo* in anaesthetised rats (Butt *et al.* 1990). Astrocyte co-culture has also been reported to increase TEER values in monolayers of hCMEC/D3 cells, but the values obtained were still low (Hatherell *et al.* 2011). Lippmann *et al*. concluded that hPSC-derived hBMECs should be a valuable tool in drug screening approaches as these cells exhibit important BBB characteristics and unlimited self-renewal capabilities (Lippmann *et al.* 2012). This method may also provide an exciting opportunity for the study of patient-specific hPSC-derived hBMECs to analyse differences in drug transport across the BBB *in vitro*.

6.3. Membrane transporter biomarkers

The clinical analysis of genetic biomarkers as predictors for treatment response or side effects represents an important alternative and additional method for the identification and clinical validation of membrane transporters involved in drug ADME. As currently available *in vitro* methods proved to be insufficient to ultimately identify the observed TPM efflux and CLP uptake processes observed in chapters 3 and 5, a clinical approach may be more successful.

The SANAD study, analysed in chapter 2 for the association of three *ABCC2* polymorphisms with CBZ treatment response, included an arm with patients randomised to TPM (Marson *et al.* 2007). The GWAS data obtained by Speed *et al*. could thus be stratified for treatment response to TPM (Speed *et al.* 2013). It remains questionable, however, if the medium effect size observed *in vitro* in chapter 3 will be sufficient to result in a statistically significant clinical effect due to the limited number of patients that consented to genotyping.

Given the large effect size observed with CLP *in vitro* in chapter 5, this is potentially a more promising drug to be analysed clinically, particularly with regard to efficacy and the severe side effects that are associated with CLP treatment (Novartis Pharmaceuticals Corporation 2013). A GWAS with samples from a CLP-treated patient cohort may be a promising strategy to identify the unknown CLP uptake transporter. There are, however, limitations to the clinical approach as well. As discussed in chapter 2 section 2.4 for the analysis of an association between *ABCC2* genetic polymorphisms and CBZ treatment response, the sample size, definition of outcome, time of observation, disease phenotype, co-treatments, prospective vs. retrospective studies, and the study population were particularly critical components to be considered in the analysis. Also, a genetic association needs to be present, an assumption that is not required for a drug to be a substrate of a given transporter. For CLP, however, the large inter-individual differences in treatment response and occurrence of side effects indicate that genetic polymorphisms of the unknown uptake transporter or receptor may indeed be present.

Ideally, clinical analysis should be combined with *in vitro* analysis to obtain the most comprehensive conclusion – this is shown by the data in chapter 2 with ABCC2 (MRP2) and CBZ. If a clinical approach is successful in identifying genetic transporter or receptor biomarker(s) associated with CLP treatment response and/or adverse effects, stably transfected cell lines with the identified gene(s) should be generated. A cell line utilising a “screen and insert” strategy, similar to the Rht14-10 cell line stably transfected with a vector coding for ABCC2 tagged to EGFP as used in chapter 2 (Arlanov *et al.* 2012, Brough *et al.* 2007), would provide

the exciting opportunity not only to confirm drug transport but to also compare the functional impact of genetic polymorphisms.

6.4. Conclusions

Membrane transporters are emerging as an important component in the complex network that determines the pharmacokinetic properties of drugs. Moreover and of particular importance, individual differences in drug transport, for example by genetic polymorphisms, may predict efficacy and/or adverse effects associated with treatment on a personalised level. Drug-drug interactions can be a direct result of two drugs competing with the same transporter, similar to that seen with CYP enzymes, and are thus critical for drug safety.

CNS diseases represent a major burden to population health, and there is a need for the development of drugs that will have to cross the BBB to act on targets in the brain. It is thus important that the passage of such drugs is carefully characterised – this will provide a more comprehensive picture of the potential of the drugs to lead to variability in both drug efficacy and drug safety.

In conclusion, this thesis demonstrates that drug transporters can play a critical role in the effectiveness and/or safety of neurological drugs. However, given the large number of SLC and ABC transporters that have not been functionally characterised yet, a lot of work is still to be done. Robust and large-scale functional screening methodologies are urgently needed for systematic and broader screening approaches in drug development, aiming for a more comprehensive understanding of drug transporters in ADME.

6.5. References

Arlanov, R.*, et al.* (2012) Functional characterization of protein variants of the human multidrug transporter ABCC2 by a novel targeted expression system in fibrosarcoma cells. *Human mutation* **33**, 750-762

Bachmann, K. (2009) Chapter 8: Drug Metabolism in *Pharmacology* (Hacker, M., Messer, W., and Bachmann, K. eds.), Elsevier Inc. pp 131-173

Brodie, M. J., Schachter, S. C., and Kwan, P. (2010) Fast Facts: Epilepsy, *Health Press Limited*

Brough, R., Papanastasiou, A. M., and Porter, A. C. (2007) Stringent and reproducible tetracycline-regulated transgene expression by site-specific insertion at chromosomal loci with pre-characterised induction characteristics. *BMC Mol Biol* **8**, 30

Butt, A. M., Jones, H. C., and Abbott, N. J. (1990) Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. *The Journal of physiology* **429**, 47-62

Carl, S. M.*, et al.* (2010) ABC and SLC transporter expression and pot substrate characterization across the human CMEC/D3 blood-brain barrier cell line. *Mol.Pharm.* **7**, 1057-1068

Chakos, M.*, et al.* (2001) Effectiveness of second-generation antipsychotics in patients with treatment-resistant schizophrenia: a review and meta-analysis of randomized trials. *The American journal of psychiatry* **158**, 518-526

David Josephy, P., Peter Guengerich, F., and Miners, J. O. (2005) "Phase I and Phase II" drug metabolism: terminology that we should phase out? *Drug metabolism reviews* **37**, 575-580

Dickens, D.*, et al.* (2012) Lamotrigine is a substrate for OCT1 in brain endothelial cells. *Biochemical pharmacology* **83**, 805-814

Dickens, D.*, et al.* (2013) Transport of gabapentin by LAT1 (SLC7A5). *Biochemical pharmacology* **85**, 1672-1683

Doring, B., and Petzinger, E. (2014) Phase 0 and phase III transport in various organs: Combined concept of phases in xenobiotic transport and metabolism. *Drug metabolism reviews*

Forster, C.*, et al.* (2008) Differential effects of hydrocortisone and TNFalpha on tight junction proteins in an in vitro model of the human blood-brain barrier. *The Journal of physiology* **586**, 1937-1949

Giacomini, K. M.*, et al.* (2013) International Transporter Consortium commentary on clinically important transporter polymorphisms. *Clinical pharmacology and therapeutics* **94**, 23-26

Giacomini, K. M.*, et al.* (2010) Membrane transporters in drug development. *Nat.Rev.Drug Discov.* **9**, 215-236

Giaever, G.*, et al.* (2002) Functional profiling of the Saccharomyces cerevisiae genome. *Nature* **418**, 387-391

Hatherell, K.*, et al.* (2011) Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models. *Journal of neuroscience methods* **199**, 223-229

Ishikawa, T. (1992) The ATP-dependent glutathione S-conjugate export pump. *Trends in biochemical sciences* **17**, 463-468

Kane, J.*, et al.* (1988) Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. *Archives of general psychiatry* **45**, 789-796

Lanthaler, K.*, et al.* (2011) Genome-wide assessment of the carriers involved in the cellular uptake of drugs: a model system in yeast. *BMC biology* **9**, 70

Lieberman, J. A.*, et al.* (1994) Clinical effects of clozapine in chronic schizophrenia: response to treatment and predictors of outcome. *The American journal of psychiatry* **151**, 1744-1752

Lippmann, E. S.*, et al.* (2012) Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. *Nature biotechnology* **30**, 783-791

Marson, A. G.*, et al.* (2007) The SANAD study of effectiveness of carbamazepine, gabapentin, lamotrigine, oxcarbazepine, or topiramate for treatment of partial epilepsy: an unblinded randomised controlled trial. *Lancet* **369**, 1000-1015

Mkrtchyan, H.*, et al.* (2009) Molecular cytogenetic characterization of the human cerebral microvessel endothelial cell line hCMEC/D3. *Cytogenetic and genome research* **126**, 313-317

Novartis Pharmaceuticals Corporation (2013) Clozaril Prescribing Information

Petzinger, E., and Geyer, J. (2006) Drug transporters in pharmacokinetics. *Naunyn-Schmiedeberg's archives of pharmacology* **372**, 465-475

Poller, B.*, et al.* (2008) The human brain endothelial cell line hCMEC/D3 as a human blood-brain barrier model for drug transport studies. *J Neurochem* **107**, 1358-1368

Speed, D.*, et al.* (2013) A genome-wide association study and biological pathway analysis of epilepsy prognosis in a prospective cohort of newly treated epilepsy. *Human molecular genetics*

United States Food and Drug Administration (2012) Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations.

Urich, E.*, et al.* (2012) Transcriptional profiling of human brain endothelial cells reveals key properties crucial for predictive in vitro blood-brain barrier models. *PloS one* **7**, e38149

Warren, M. S.*, et al.* (2009) Comparative gene expression profiles of ABC transporters in brain microvessel endothelial cells and brain in five species including human. *Pharmacol Res* **59**, 404-413

Weksler, B., Romero, I. A., and Couraud, P. O. (2013) The hCMEC/D3 cell line as a model of the human blood brain barrier. *Fluids and barriers of the CNS* **10**, 16

Weksler, B. B.*, et al.* (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 1872-1874

Williams, R. T. (1959) Detoxication Mechanisms: The Metabolism and Detoxication of Drugs, Toxic Substances, and Other Organic Compounds, 2nd edition, *Chapman and Hall*

World Health Organisation (2009) Epilepsy fact sheet no. 999 http://www.who.int/mediacentre/factsheets/fs999/en/index.html

Appendix

Table 1: TaqMan® gene expression assays supplied by Life Technologies Ltd., Paisley, UK. GAPDH = glyceraldehyde- 3-phosphate dehydrogenase; MGBNFQ = Minor groove binder nonfluorescent quencher

Gene	Reporter dye	Quencher	Catalogue number, Assay ID
GAPDH	VIC®	TAMRA™	4310884E
ß-actin (ACTB)	FAM™	MGBNFQ	4352935E
ABCC1	FAM™	MGBNFQ	4331182, Hs00219905_m1
ABCC2	FAM™	MGBNFQ	4331182, Hs00166123_m1
ABCC3	FAM™	MGBNFQ	4331182, Hs00978473_m1
ABCC4	FAM™	MGBNFQ	4331182, Hs00988717_m1
ABCC5	FAM™	MGBNFQ	4331182, Hs00981087_m1
ABCC6	FAM™	MGBNFQ	4331182, Hs00184566_m1
ABCC7	FAM™	MGBNFQ	4331182, Hs00357011_m1
ABCC8	FAM™	MGBNFQ	4331182, Hs01093761_m1
ABCC9	FAM™	MGBNFQ	4331182, Hs00245832_m1
ABCC10	FAM™	MGBNFQ	4331182, Hs00375701_m1
ABCC11	FAM™	MGBNFQ	4331182, Hs01090768_m1
ABCC12	FAM™	MGBNFQ	4331182, Hs00264354_m1
SLC6A2	FAM™	MGBNFQ	4448892, Hs00426573_m1
SLC6A3	FAM™	MGBNFQ	4448892, Hs00997364_m1
SLC6A4	FAM™	MGBNFQ	4448892, Hs00984349_m1
SLC7A5	FAM™	MGBNFQ	4331182, Hs00185826_m1
SLC22A1	FAM™	MGBNFQ	4331182, Hs00427552_m1
SLC22A2	FAM™	MGBNFQ	4331182, Hs01010723_m1
SLC22A3	FAM™	MGBNFQ	4331182, Hs01009568_m1
SLC22A4	FAM™	MGBNFQ	4331182, Hs01548718_m1
SLC22A5	FAM™	MGBNFQ	4331182, Hs00929869_m1
SLC28A1	FAM™	MGBNFQ	4448892, Hs00984403_m1
SLC28A2	FAM™	MGBNFQ	4448892, Hs00188407_m1
SLC28A3	FAM™	MGBNFQ	4448892, Hs00910439_m1
SLC29A1	FAM™	MGBNFQ	4448892, Hs01085704_g1
SLC29A2	FAM™	MGBNFQ	4453320, Hs00155426_m1
SLC29A3	FAM™	MGBNFQ	4448892, Hs00217911_m1
SLC29A4	FAM™	MGBNFQ	4448892, Hs00928283_m1

Table 2: Human siGENOME SMARTpool siRNAs and control, 5 nmol, supplied by Fermentas GmbH – Thermo Fisher Scientific, St. Leon-Rot, Germany

Gene	Target sequences 5' -> 3'	Catalogue number
ABCC1	GAUGACACCUCUCAACAAA UAAAGUUGCUCAUCAAGUU CAACGAGUCUGCCGAAGGA CGAUGAAGACCAAGACGUA	M-007308-01-0005
ABCC2	GAAUCAAGAUCCUGAAAUA GAACCUGACUGUCUUCUUU CUACGGAGCUCUGGGAUUA CAUUGUGGCUGUUGAGCGA	M-004225-01-0005
ABCC3	CAAAGAAUGUCGACCCUAA GCUCUUUGCUGCACUAUUU GCACGCCGCUCUUCACUGU GAUCAUGGGUGUCAUCUAC	M-007312-00-0005
ABCC4	GACCAUGAAUGAAGUUAUA UCUGAAAGCUCCGGUAUUA GAUCUUAACUGGUACUUAG GGGCAGAAAGCACGGGUAA	M-007313-01-0005
ABCC5	CGACAUAGGAAAAGAGUAU UGCGAAGGGUUGUGUGGAU AGAACUCGACCGUUGGAAU CUGAAGCCCAUCCGGACUA	M-007314-02-0005
ABCC6	GAACAAUGCUCGCGUAGAU GAGCAAAGCCCACCUCAGU GGACGACCCUGCAGUAGGU GGGCUAAGAUACCGACCUG	M-007315-01-0005
ABCC10	GCAGAGCCAUCUACAGUAU GUUGUCAUCUCCAUCGUUA CGGCAAGUCUUCCCUGUUG UGAGUGAGGUGAUUACAUC	M-007309-00-0005
SLC22A3	GGACGUGGAUGACUUGCUA CCAACAACAUUACGAAAUU GCACCAAACUUCCCUGUGU GGAUAACCAUGGCCUUUGA	M-007454-00-0005

Gene	Target sequences 5' -> 3'	Catalogue number
SLC22A5	CCAUAUCAGUGGGCUAUUU CGUCUUAUGUAUGGUUUAA CAGAGGUGAUCAUCCGCAA GGAAGAAUGUGCUGUUCGU	M-007456-02-0005
SLC28A3	GGAAACAACUCAAUAAGAA GGGAUCAUCUCCUAUUGAA UAAACGGUGUGCAGCAAUA CAAAUACGAACAUCGAAUU	M-007505-01-0005
SLC29A1	GAAAGCCACUCUAUCAAAG GCCAUGAUCUGCGCUAUUG AGAGGAAUCUGGAGUUUCA UCAAGAUCGUGCUCAUUAA	M-003709-01-0005
SLC29A2	GCUACUACCUGGCCAAUAA CCUCCAGUCUGAUGAGAAC GCUACCACCUGGUCGGGAU CGGAAUCAGAGCCAGAUGA	M-007506-00-0005
SLC29A3	CAACUUUGCUGACCUAUGU CCAUGGAACUUCUUUAUCA UCACUACCUUCCUCCUGUA CGUAAUAAAUACUUGCGUA	M-007507-01-0005
SLC29A4	CGCUGGAGCUGCUGUGUUU GCUUCACCUUCGACAGUCA ACGUGUGGCUGCAGCUCUU GGAUGCUGCCCAAGCGGUA	M-007508-01-0005
Non-targeting siRNA pool #2	Not disclosed by manufacturer	D-001206-14-05

Table 3: FlexiTube GeneSolution siRNAs for human *ABCC5* and control, 1 nmol and 5 nmol, respectively, supplied by Qiagen Ltd., Manchester, UK

Gene	Target sequences 5' -> 3'	Catalogue number
ABCC5	ACCGTGAAGATTCCAAGTTCA ATGAAGGATATCGACATAGGA CCCATCCGGACTACTTCCAAA GACGTGAACTGCAGAAGACTA	GS10057
AllStars negative control siRNA	Not disclosed by manufacturer	1027280

Table 4: Silencer® Select siRNA for human *SLC7A5* and control, 5 nmol, supplied by Life Technologies Ltd., Paisley, UK

Gene	siRNA sequence sense strand 5' -> 3'	Catalogue number
SLC7A5	UGUCCAAUCUAGAUCCCAAtt	4392420, ID s15653
Negative control no. 1	Not disclosed by manufacturer	4390843