ABC TRANSPORTERS AND DRUG RESISTANCE IN PATIENTS WITH EPILEPSY

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INTRODUCTION

Epilepsy is a common neurological disorder with an estimated prevalence in Europe of 4.3-7.8 per 1000 [1]. Despite advances in antiepileptic drug (AED) therapy about one-third of patients with epilepsy are resistant to drug treatment [2]. Most patients with refractory epilepsy are resistant to several, if not all, AEDs, even though these drugs act by diverse mechanisms [3].

Temporal lobe epilepsy (TLE) is the most common focal epilepsy. The most common cause of TLE in surgical series is hippocampal sclerosis (HS), which can be reliably detected in vivo by magnetic resonance imaging (MRI) [4]. Structural damage in TLE associated with HS is a condition that characterises mesial temporal lobe epilepsy (mTLE). Such damage and dysfunction frequently extends beyond the hippocampus into the parahippocampal and entorhinal cortex [5]. In this review we will first describe one putative mechanism of pharmacoesistance in epilepsy, the transporter hypothesis, and then discuss preclinical and clinical studies investigating ABC transporters and pharmacoresistance in epilepsy and emphasize molecular imaging techniques as a help towards analysis of the mechanisms of pharmacoresistance in epilepsy.

PHARMACORESISTANT EPILEPSY

Definition of pharmacoresistant epilepsy

The concept of pharmacoresistant epilepsy seems to be self-explanatory, but for some years a universally agreed definition remained elusive. In 2010 the International League against Epilepsy suggested that pharmacoresistant epilepsy be defined as failure of adequate response to two tolerated and appropriately prescribed AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom [6].

There are several hypotheses that explain the mechanisms associated with pharmacoresistance in epilepsy. Current theories on the causes of pharmaco-resistance in epilepsy include the transporter hypothesis, the target hypothesis, the network hypothesis, the gene variant/methylation hypothesis and the intrin-sic severity hypothesis. However, none of these hypotheses is currently a stand-alone theory that is able to convincingly explain how drug resistance arises in human epilepsy [7].

The transporter hypothesis

The drug transporter hypothesis proposes that pharmacoresistance is related to increased expression of multidrug efflux transporter proteins such as P-glycoprotein (Pgp). These proteins are thought to prevent AED entry by actively extruding AEDs from their target site [8]. Multidrug efflux transporters are highly expressed in capillary endothelial cells and astrocytic foot processes that form the Blood-Brain Barrier (BBB). They limit intracellular concentration of substrates

by pumping them out of the cell through an active energy-dependent mechanism. Pgp (encoded by the adenosine triphosphate (ATP)-binding cassette subfamily B member 1 gene (ABCB1) was discovered more than thirty years ago [9] and it is the multidrug efflux transporter protein we know most about in terms of its structure and mechanism. Epilepsy was the first CNS disorder for which pharmacoresistance was associated with enhanced expression of Pgp in the brain [10]. Pathologically elevated expression of Pgp has been found in resected brain tissue of patients with pharmacoresistant mTLE undergoing surgery [8] as well as in limbic brain regions of mouse and rat models of mTLE [11]. It is currently not clear whether the endothelial and parenchymal overexpression of Pgp is a consequence of epilepsy, of uncontrolled seizures, of chronic treatment with AEDs, or is constitutive, i.e. present before the onset of epilepsy [11].

Blood-brain barrier
The blood-brain barrier (BBB) is a physical and metabolic barrier between the brain and the systemic circulation [12]. The BBB is composed of a monolayer of brain capillary endothelial cells. Unlike capillaries in other parts of the body, the cerebral capillaries are joined by tight junctions, which restrict solute flux between the blood and the brain. The brain capillary endothelial cells are surrounded by extracellular matrix, pericytes, and astrocyte foot processes [13]. Circulating molecules gain access to the brain via one of two processes: 1) lipid-mediated transport of small nonpolar molecules through the BBB by free (passive) diffusion, or 2) catalyzed transport [12, 13].

The endothelial cells of the BBB contain numerous membrane transporters involved in the influx and efflux of essential substrates [14]. ABC efflux transporters, such as Pgp, at the BBB limit the brain uptake of a variety of therapeutic agents, including compounds that are relatively lipophilic and would be predicted to permeate the endothelial lining of the brain microvasculature [15]. Pgp is located at the luminal (apical = blood-facing side) membrane of endothelial cells [16, 17]. Thus, Pgp substrates entering the endothelial cells from blood are immediately pumped back into blood. As a consequence, the net penetration of substrate compounds from the blood into the brain tissue can be dramatically decreased. In the absence of Pgp in the BBB, the brain penetration of Pgp substrate drugs can increase up to ten - to 100-fold, with sometimes dramatic consequences for the toxicity of compounds [16]. Furthermore, blockade of BBB Pgp by cerebral application of Pgp inhibitors significantly increases the brain concentration of various drugs, again being in line with Pgp functioning as an efflux transporter in the BBB [18].

Clear expression of Pgp in astrocytes is especially seen in certain pathological states such as epilepsy [19].

In contrast to Pgp, data on the other ABC transporters in the BBB are much more limited [16]. At least six multidrug resistant proteins (MRP) (MRP1-6) are expressed at the BBB of different species. However, the exact subcellular localization (apical vs basolateral) of most of these MRPs in brain capillary endothelial cells remains to be determined [11].

Blood-cerebrospinal-fluid barrier

Several drug efflux transporters are present in epithelial cells of the blood-cerebrospinal-fluid (CSF) barrier. However, in contrast to the BBB, the exact functional role of efflux transporters in these other brain barrier is less well understood [18]. The blood-CSF barrier is located at the choroid plexus (CP) and the outer arachnoid membrane. The blood-CSF barrier plays a vital role in the selectivity and permeability of the CP membrane to various nutrients and xenobiotics [14]. The CP, which is the main source for CSF production, is a leaf-like highly vascular organ that projects into the ventricles of the brain and functions as a highly regulated solute- and drug-permeability barrier. The apical surface area has a same size range as the luminal surface area of the endothelial cells of the BBB, thereby providing a similarly large surface for solute exchange [18]. The CP is comprised of fenestrated, highly permeable capillaries at the blood side that are surrounded by stroma and a monolayer of epithelial cells that face the CSF and are joined together by tight junctions [14]. Once a solute or drug has crossed the capillary wall, it must penetrate the epithelial cells before entering the CSF [18].

The molecular identity and localization of the proteins responsible for the influx and efflux of drugs and metabolites at the CP are currently explored. Transport of drugs and metabolites involves mainly the solute carrier and ABC transporters [14, 20, 21]. Rao et al. [22] first described the expression of Pgp and MRP1 in the epithelia of the CP and their contribution in a bipolar permeation barrier for selected drugs crossing the blood-CSF barrier [18]. It was concluded that Pgp

localizes sub-apically at the CP with transport into the direction of CSF, whereas MRP1 localizes basolaterally, conferring transport to the blood side of epithelial cells [22]. Thus, in the choroidal epithelium, Pgp and MRP1 seem to have opposing drug-transport functions. The basolateral localization of MRP1 at the CP epithelium was subsequently confirmed by other groups [23]. However, in general, the role of these and other drug transporters in blood-CSF barrier function is only incompletely understood and more studies need to be done [18].

ABC transporters: function and role in pharmacoresistance

Active drug efflux transporters of the ATP binding cassette (ABC)-containing family of proteins have a major impact on the pharmacological behaviour of most of the drugs in use today. The penetration of drugs into a range of important pharmacological sanctuaries, such as brain, testis, and fetus, and the penetration into specific cell- and tissue compartments can be extensively limited by ABC transporters [16, 24]. ABC transporters are expressed in many tissues including the intestine, liver, kidney, brain and they maintain chemical homeostasis by mediating the transport of molecules across a membrane irrespective of concentration gradient. These transporters are encoded by 49 genes in the human genome and have been grouped into seven subfamilies (designated ABCA-ABCG) based on sequence homology [25-27]. ABC transporters in their functional form comprise a minimum of four core domains that form the permeation pathway for transport of substrates, and two nucleotide binding domains that hydrolyze adenosine triphosphate (ATP) to power this process. Three ABC proteins appear to account for most observed multidrug

resistance (MDR) in humans and rodents [28]; P-glycoprotein (Pgp/MDR1/ABCB1), MDR-associated protein (MRP)1 (ABCC1) and breast cancer resistance protein ABCG2 (variously known as BCRP, ABCP for its high expression in placenta or MXR for mitoxantrone resistance) [29]. We will focus on these three most important multidrug transporters; however the family of mammalian ABC transporters is far more extensive and functionally highly diverse [30].

P-glycoprotein

Pgp was discovered more than 30 years ago [9] and it is the ABC protein we know most about in terms of its structure and mechanism. Pgp is generally expressed at higher levels in epithelial cell surfaces throughout the body. It is found exclusively at the apical surface of cells in the kidney proximal tubule, canalicular membrane of hepatocytes, pancreas, the villous membrane of the small and large intestine and the adrenal gland [31, 32]. Pgp is also located in blood-tissue barriers, including the placenta and endometrium, blood-inner ear barrier, blood-mammary tissue barrier, blood-testis barrier, blood-nerve barrier, blood- brain barrier (where it is exclusively oriented to transport substrates toward blood) and epithelial cells of the blood-CSF barrier [22, 33]. Pgp either restricts drug-entry to the body via the gastrointestinal tract and excretes metabolites into the urine or gastrointestinal tract or prevents their access from the blood to the fetus and sensitive organs such as the brain and testis [33].

Pgp is a phosphorylated glycoprotein with an apparent molecular weight of 170kDA [18]. There are two types of human Pgp: type I, encoded by the MDR 1

gene (ABCB1), which confers the drug resistance phenotype and promotes drug efflux at the blood-brain barrier, and type II, encoded by (ABCB2), present in the canalicular membrane of hepatocytes and functioning as phosphatidylcholine translocase [34]. The MDR1 gene in humans is located on chromosome seven and has 28 exons [35]. Structurally the transporter consists of two interwoven transmembrane regions, each containing six transmembrane helices and an ATP-binding site located intracellularly. The transmembrane helices of Pgp allow it to bind and induce efflux of a broad range of substrates with varying affinities [36]. Although substrates for Pgp tend to be hydrophobic or weak base molecules with a planar ring system, Pgp is considered polyspecific because it can recognize a wide range of substrates including antiarrhythmics, antihistamines, cholesterol-lowering statins and human immunodeficiency virus protease inhibitors [26].

Multidrug resistance protein

The multidrug resistance protein (MRP) 1 was discovered in 1992 by Cole [37] and co-workers and the MRP family of proteins comprised nine characterized members (MRP1-9) also named ABCC1-6 and 10-12, respectively. Among MRP proteins, MRP1 is the most studied and like Pgp, MRP1 is an ATP-dependent transporter. It is expressed at low levels throughout many normal tissues and cell types in the body, but it is more highly expressed in the adrenal gland, bladder, CP, colon, erythrocytes, kidney, lung, placenta, spleen, stomach, testis, helper T-cells and muscle (both skeletal and cardiac) [33]. In contrast to Pgp and MRP2, it is localized on the basolateral membranes in polarized cells [38].

The basolateral localization of MRP1 serves to protect sensitive tissues. For example, basolateral expression of MRP1 in the CP allows the protein to transport drugs from the cerebrospinal fluid to the blood to protect sensitive nervous tissues [22]. MRP1 is a 190-kDa protein containing 1531 amino acids and its cognate gene is located on chromosome 16p13.11 [39]. MRP1 functions mainly as a co-transporter of amphipathic organic anions. It can transport hydrophobic drugs or other compounds that are conjugated or complexed to the anionic tripeptide glutathione, glucuronic acid, or to sulfate [16].

Breast cancer resistance protein

In the early 1990s several groups began reporting non-Pgp, non-MRP1-mediated pharmacoresistance in a variety of drug selected cell lines [40, 41]. The gene responsible for the novel phenotype was first cloned by Doyle and colleagues from a breast cancer cell line and was therefore called BCRP for breast cancer resistance protein [42]. Like Pgp, the breast cancer resistance protein (BCRP) is also localized to the apical face of polarized membranes. It is found in epithelial cells of the intestine, human placenta syncytiotrophoblasts, liver bile canaliculi, prostate, brain, lobules and lactiferous ducts of the mammary gland, and renal tubules, as well as the endothelium of veins and capillaries, including those at the blood-brain barrier and the placenta [33, 42]. Based on messenger ribonucleic acid (mRNA) analysis ABCG2 was more strongly expressed at the BBB than Pgp or MRP1 [43].

The tissue distribution of BCRP shows extensive overlap with that of Pgp, suggesting that it might have a similar role as Pgp in the pharmacological

handling of substrate drugs. It appears to transport both positively and negatively charged drugs, including sulfate conjugates and the list of its substrates is rapidly expanding, highlighting the importance of this protein [28].

MECHANISMS OF PGP OVEREXPRESSION IN PHARMACORESISTANT EPILEPSY

An important question is whether the overexpression of efflux transporters in epileptic brain tissue is constitutive or acquired/induced, or both mechanisms may be at play. A constitutive overexpression could occur as a result of a genetic predisposition, or it could be intrinsic to the development of the specific pathology. It is also conceivable that overexpression is acquired such as induction by recurrent seizures or even the AEDs intended to prevent them [3]. We will discuss the evidence for the possible various mechanisms in the following paragraphs.

Seizures induce Pgp overexpression

In rats and mice, experimentally induced seizures have been shown to increase the expression of Pgp in brain capillary endothelial cells (BCECs), astrocytes and neurons [18]. Zhang et al. [44] reported increased Pgp expression in the hippocampus following intracerebroventricular injection of kainate in rats, causing neuronal injury. This increase in Pgp expression was observed in reactive astrocytes as early as one day after injection, peaked at two weeks but was still visible at ten weeks. Seeger et al. [45] studied Pgp expression at 24 hours and ten days after status epilepticus (SE) (to differentiate between seizure related changes and changes developing during epileptogenesis). Pgp

increased significantly 24 hours after kainate-induced SE in BCECs at the dentate gyrus, amygdala, piriform and parietal gyrus. Additionally in the brain parenchyma of rats 24 hours after SE, a significant increase in Pgp expression was observed in the piriform and parietal cortex, dentate gyrus and hippocampus. However, the alterations in Pgp expression were only transient and disappeared ten days after the SE, except for the dentate hilus and the CA1 sector of the hippocampus, in which a significant increase in parenchymal Pgp was observed ten days after the status. Following systemic injection of kainate in mice, expression of mdr-1, the gene encoding Pgp, was found to be increased in the hippocampus for three-24 hours after the seizures but returned to control level by 72 hours [46]. Recently Pekcec et al. [47] studied Pgp expression in brain tissue sampled from epileptic dogs following spontaneous status epilepticus or seizure clusters to avoid a putative bias of status epilepticus induction. They demonstrated a significant upregulation of Pgp in the hippocampal hilus (82% above control), the dentate gyrus granule cell layer (132% above control) and parietal cortex of canines (123% above control) one-seven days following a seizure. In further support of the temporal increase of Pgp after seizures is the study by Bankstahl and co-workers [48] in two rat SE models. Immunohistochemical staining of Pgp did not indicate any increase of Pgp expression in brain capillary endothelial cells during SE, whereas significant overexpression was determined in both models 48 hours after SE. Seven days after SE, Pgp expression had returned to control levels [48]. Löscher and Potschka [11] suggested that the excitatory neurotransmitter glutamate, which is excessively released by seizures, is involved in the seizure-induced over-

expression of Pgp in the brain. This suggestion was based on a report by Zhu and Liu [49], showing that glutamate up regulates the expression and functional activity of Pgp in rat BCECs in vitro. They suggested that glutamate upregulates Pgp expression in BCECs by an N-methyl-D-aspartate (NMDA) receptor-mediated mechanism, which could play a role during ischemic and anoxic injury. Bankstahl et al. [50] recently examined this hypothesis and evaluated whether glutamate is involved in seizure-induced upregulation of Pgp in brain capillaries after systemic-administration of pilocarpine, a model of mTLE. Their data shows that the administration of the glutamate receptor antagonist MK-801 after convulsive SE prevents the upregulation of Pgp in brain microvessels in the hippocampus, indicating that SE-induced glutamate release is involved in the regulation of Pgp expression after seizures. Additionally MK-801 reduces the neuronal damage after prolonged seizures which may offer a therapeutic option. The activation of the NMDA receptor by glutamate is known to generate reactive oxygen species (ROS) and Zhu and Liu [49] suggested that ROS may mediate the effect of glutamate on Pgp expression. Generation of free radicals such as ROS has been suggested to play a key role in neuronal damage developing after SE. It is generally believed that excitotoxic cell death is due to excessive activation of NMDA receptors by glutamate, leading to excessive activation of calcium ion influx through the receptor's associated ion channels and subsequent free radical production, including ROS. Based on this hypothesis, ROS is likely to be the link between seizures induced glutamate release and over-expression of Pgp. The data from these animal studies indicate that Pgp

overexpression is a result of sustained seizure activity but not of the processes underlying development of epilepsy.

Brain inflammation and epilepsy

It is widely accepted that neuronal dysfunctions are the cause of seizures and targeting of neuronal ion channels, GABA, and glutamate receptors has been, for decades, the mainstream pharmacological approach to eradicate seizures. Although the ultimate effectors of seizures are neurons, recent advances in experimental neurology have revealed that inflammation can precipitate seizures or sustain seizure activity [51]. Two distinct inflammatory processes have been linked to seizures. Neuroinflammation is present in epileptic brain where it exacerbates seizures or increases their frequency [52]. By contrast, systemic inflammation can cause epileptiform neuronal discharge via loss of ionic (e.g., potassium [53]) and neurotransmitter (e.g., glutamate [51, 54]) homeostasis. Although neuroinflammation directly affects neurovascular and glial function, the effects of systemic inflammation are mediated or facilitated by loss of BBB function [55]. BBB disruption can be triggered by a direct insult to the endothelium [56] or by systemic factors, including activation of circulating leukocytes and release of molecular mediators that increase vascular permeability [51, 57]. Pro-inflammatory and anti-inflammatory cytokines, chemokines, and prostaglandins are responsible for the production of an early immune response. Numerous studies have confirmed enhanced inflammatory signalling in chronic rodent models and in tissue from patients with TLE [58, 59]. Genetic and pharmacological modulation of the synthesis and secretion of

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inflammatory mediators and of their receptors can influence seizure thresholds, severity and duration in mouse and rat TLE models [59]. Anti-inflammatory drugs, such as steroids and intravenous immunoglobulins, are useful in selected pharmacoresistant epileptic syndromes, whereas fever, immunization, and trivial infection can precipitate seizures, providing a solid link between inflammation and seizures [60]. In addition, inflammatory mediators seem to contribute to disease-associated alterations, which can exert an effect on antiepileptic drug responses. For instance, cytokines, such as interleukin (IL) - IL-1 β,IL-2, IL-6 and tumor necrosis factor- α (TNF- α), can modulate the expression, sub-unit composition and functional state of antiepileptic drug targets [59, 61, 62].

Pgp overexpression and brain inflammation

Further downstream events in the signalling cascade have been identified and Bauer et al [63] have shown that cyclooxygenase-2 (COX-2) is a central factor of a cascade that drives the transcriptional activation of the Pgp-encoding gene in the epileptic brain. COX is an enzyme that is responsible for the formation of prostanoids, including prostaglandins, prostacyclin and thromboxane. These lipid mediators play important roles in inflammation and pain and in normal physiological functions. Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain. It has been proven that COX-2 mediates Pgp regulation in response to excess glutamate concentrations such as those occurring during epileptic seizures [64]. It is known that glutaminergic signaling increases COX-2 expression and that at least in rat mesangial cells COX-2 activation leads to increased Pgp [65]. Furthermore, both the

nonselective COX inhibitor indomethacin as well as the selective COX-2 inhibitor celecoxib counteracted Pgp increase by glutamate exposure of isolated rat brain capillaries. Van Vliet et al. tested the efficacy of COX-2 inhibitors in a chronic rodent model, overexpressing Pgp [66, 67]. A sub-chronic 2-week treatment with the highly selective COX-2 inhibitor SC-58236 in the chronic epileptic state kept Pgp expression at control levels [68]. These data substantiate that COX-2 inhibition can block repeated induction of Pgp by ongoing seizure activity, thereby allowing Pgp to return to control levels. Furthermore, enhanced Pgp expression in chronic epileptic rats was associated with a significant reduction in the brain penetration of the antiepileptic drug phenytoin. Importantly, the brain delivery of phenytoin was significantly enhanced by subchronic COX-2 inhibition in rats with recurrent seizure activity [68]. These data provided evidence that COX-2 inhibition may help to increase concentrations of AEDs at the target sites and that COX-2 inhibition could be a novel therapeutic concept to overcome pharmacoresistance in epilepsies [69].

Genetic polymorphism and Pgp expression

Several studies have tested for an association between Pgp expression in patients with epilepsy and polymorphism in drug transporter genes [70]. One of these, a single nucleotide polymorphism in exon 26 (C3435T) of the MDR1 gene is associated with altered expression, functionality and substrate specificity of the MDR1 product Pgp. Based on an initial report by Siddiqui [71], a series of studies supported the hypothesis that the C3435T polymorphism is associated with resistance to multiple AEDs [72]. Several recent genetic association studies

have also indicated an association of the 3435CC genotype with increased Pgp expression and drug resistant epilepsy [72]. In contrast however are other studies which did not find a link between ABCB1 and response to AEDs [72]. Two metaanalyses including 11 studies up to September 2007 involving 3371 patients and including 22 studies up to February 2010 involving 6755 patients respectively provided no support for an association between pharmacoresistance and ABCB1 [73, 74]. The inconsistency between the studies might be caused by different inclusion criteria (definition of pharmacoresistance, co-morbidities, co-medication and population structure). A recent study pointed out the importance of stratification by patient age and aetiology of epilepsy [75]. Another issue is the selection of AEDs included in the studies as the inclusion of drugs which have not been confirmed as Pgp substrates will bias the data [69]. Another factor complicating genetic association studies is related to data indicating that the C3435T polymorphism might have contrasting consequences on Pgp expression in different ethnic subgroups. This is in line with the observation that an association in the opposite direction has been described in studies in Asian patients as compared to studies reporting an association in a Caucasian patient population [76].

Are antiepileptic drugs Pgp substrates?
A central question of the transporter hypothesis is whether AEDs are substrates for Pgp. Only then, could overexpression of Pgp crucially contribute to pharmacoresistance in epilepsy. So far evidence has been reported that phenytoin, phenobarbital, lamotrigine, levetiracetam, topiramate, and

carbamazepine and oxcarbazepine metabolite are substrates of human Pgp [77-79]. On the other hand data argue against a transport of valproic acid and carbamazepine [79, 80]. In addition, transport of AEDs has been evaluated in a human colon carcinoma cell line. In these in vitro assays no evidence was obtained that carbamazepine, vigabatrine, gabapentin, phenobarbital, or lamotrigine are substrate of Pgp [81, 82]. Moreover, Rivers et al. reported that carbamazepine, valproic acid, phenytoin, lamotrigin, and primidone are not likely to be substrates of Pgp based on their investigations in breast and cancer cell lines [83]. Although in vitro and in vivo transport assays have indicated that several antiepileptic drugs are substrates of Pgp, and that some AEDs are transported by ABC transporters, the data however is controversial. Although apparently a simple question, obvious difficulties exist in research evaluating the transporter substrate characteristics of AEDs. AEDs can pass the BBB efficaciously when efflux transporters such as Pgp are expressed at basal level. However, their brain penetration is limited once a relevant over expression occurs. Highly lipophilic compounds can rapidly diffuse through membranes of endothelial cells and might be less sensitive to an impact of active transports. Furthermore differences in the affinity to the transporter molecule might exist. Respective differences need to be taken into consideration when choosing an in vitro assay to explore the substrate specificities of AEDs. It has also been recently demonstrated that the transport of AEDs depends on concentration and it is therefore of particular relevance to test potential Pgp substrates at clinically relevant concentrations [84]. Additionally, recent data also suggests that the substrate recognition or transport efficacy by Pgp differs between human and

mouse for certain AEDs [85, 86]. Such differences might explain in part the controversial data which have been reported for AED transport by Pgp from different species [3].

Antiepileptic drugs induce Pgp overexpression

Some studies have indicated that antiepileptic drugs might contribute to the induction of Pgp overexpression [69]. Lü et al. [87] showed in astrocyte cell cultures from postnatal Wistar rats that the antiepileptic drugs phenytoin, phenobarbital, carbamazepine, and valproic acid induced the overexpression of Pgp in astrocytes in a dose- and time-dependent manner. Significantly higher levels of Pgp staining were detected at therapeutic concentrations of certain antiepileptic drugs (20 microg/ml phenobarbital, 40 microg/ml phenobarbital, and 20 microg/ml phenytoin) on day 30. Upregulation of Pgp was detected when using higher concentrations of phenytoin, phenobarbital, and valproic acid on day 20 and when using higher concentrations of any of the four antiepileptic drugs on day 30. Similarily, Lombardo et al. [88] reported that carbamazepine, phenobarbital, and phenytoin induce Pgp and other transporters in rat brain endothelial cell lines via and interaction with the pregnane x receptor and the constitutive androstane receptor. In contrast Ambroziak el al. [89] did not observe effects of these AEDs on expression and functionality of Pgp. Therefore definite conclusions regarding the impact of AEDs require further future efforts [69].

PRECLINICAL STUDIES OF PGP IN MTLE

Preclinical epilepsy models

Knockout mice and natural mutants

Investigations in genetically deficient animals, which lack a functional form of one or more drug efflux transporter, have contributed to a significant extent to the current knowledge about the physiological and pharmacological function of these transporters. Genetically deficient mice have been generated by knockout technologies with the purpose of studying the role of specific transporters. Furthermore, subpopulations of animals with spontaneous mutations in multidrug transporter genes have been identified for different species. The in vivo impact of Pgp in the BBB has been intensely studied in knockout mice lacking the Pgp isoform mdr1a (mdr1a (-/-) mice) or mdr1a and mdr1b Pgp (md1a/1b (-/-) mice). Mdr1a knockout mice lack the mdr1a isoform of Pgp all over the body and in the brain capillary endothelial cells. The animals are viable and fertile but are more susceptible to developing a severe, spontaneous intestinal inflammation [11].

Animal models of epilepsy

Animal models of epilepsy are a valuable tool to study models of seizure onset, neurologic changes during seizures, and new pharmacologic tools for seizure propagation or pharmacoresistance. For epilepsy research various animal models are available, each with their specific characteristics. Globally, these models are categorized into models of seizures and those of epilepsy [90]. Examples of seizures, or acute, models are the cortical stimulation model and

the maximal electroshock model. The amygdala kindling model is also an acute model, as most animals do not develop spontaneous epilepsy. The models of epilepsy, chronic models, can be subdivided into models of genetic epilepsy and models of acquired (symptomatic) epilepsy [91]. The first category includes both animals with spontaneous mutations and animals with induced mutations, resulting in epileptic symptoms and behavior. In animals of the acquired or symptomatic epilepsy models, status epilepticus (SE) is induced by electrical stimulation (amygdala, perforant path, and hippocampus) or through the administration of chemical convulsants (pilocarpine or kainic acid) in previously non-epileptic rats, which results in the development of spontaneous seizures after a latent period of days to weeks [92].

Kindling model

Since its introduction by Goddard in 1967 [93], the kindling model has been used extensively as an animal model of epilepsy. Kindling can be induced by the repeated administration of a mild electric stimulus to the rat brain via an implanted electrode into a limbic structure such as the amygdala, hippocampus, entorhinal cortex or other brain areas. Over a period of several stimulation session the rat reliably displays stage five seizures, according to the Racine scales [94]. The pathophysiology of kindling is very similar to that of human mTLE. For example, kindling leads to structural and functional changes characterized by neuronal cell loss, gliosis, neurogenesis and mossy fiber spouting [95, 96].

Pilocarpine model

The pilocarpine and the kainic acid model are probably the most commonly studied chemical-inductive models for mTLE. Pilocarpine, a potent muscarinic cholinergic agonist, is administered as a single high dose (300-380mg/kg) to rats or mice. It acutely induces sequential behavioral and electrographic changes, indicative of sustained epileptic activity, resulting in widespread damage to the forebrain. After 15-25 minutes this results in motorlimbic seizures and leads to SE within 50-60 minutes after pilocarpine administration that last for up to 12 hours. After a silent period of a few days animals start exhibiting spontaneous recurrent seizures. Morphological analysis of the brain after pilocarpine-indcued SE demonstrates cell loss in the hippocampal subfields CA1 and CA3 and in the hilus of the dentate gyurs, in the septum, olfactory tubercle, amygdala, piriform cortex, neocortex and thalamic nuclei [97, 98].

Kainic acid model

The excitotoxic glutamate analogue kainate can be systemically or intracerebrally injected into an animal and rapidly produce acute seizures. In rodents, large doses of the drug induce severe acute seizures with subsequent SE, which is followed by a quiescent period of usually several weeks [99]. This latent period is followed by the development of spontaneous recurrent seizures. Injections of kainic acid were shown to lead to cell death in the hippocampus, amygdala, entorhinal cortex and medial thalamic nuclei [100].

Preclinical studies of Pgp in epileptic rats

In animals, models of temporal lobe epilepsy, such as the kindling and kainate models, have been used to study the molecular mechanisms of pharmacoresistance. In these models a large group of kindled or epileptic rats is treated with AEDs. Subsequently subgroups of animals that either respond (pharmacosensitive/seizure-free) or do not respond (pharmacoresistant) to AEDs are selected. Pgp is overexpressed in endothelial cells and ectopically in astrocytes, after induction of sustained limbic seizures in rodents [101]. Upregulation of the mRNA of the MDR1 gene was detected in rat brain during both acute and spontaneous seizures caused by status epilepticus. More recently Volk and Löscher [101] used a rat model of temporal lobe epilepsy to examine whether Pgp expression differs in AED responders from non-responders rats. In this model, spontaneous recurrent seizures develop after status epilepticus is induced by prolonged electrical stimulation of the basolateral amygdala. They showed that phenobarbital-resistant epileptic rats exhibit significantly higher endothelial expression of Pgp in limbic brain regions compared to drug-responsive rates providing further support for the hypothesis of Pgp overexpression in pharmacoresistant epilepsy. Furthermore in another study with amygdala epileptic kindled rats significant upregulation of Pgp was reported in brain capillary endothelial cells of limbic brain regions. In these rats, brain-to-plasma concentration ratios of phenytoin in the hippocampus were about 30% lower than those measured in control rats [102]. When kindled rats were divided into phenytoin responders and non responders, non responders exhibited a significantly higher expression of Pgp in capillary endothelial cells in

the epileptogenic focus [103]. Moreover, no differences in Pgp expression were observed in the adjacent piriform cortex indicating that the Pgp expression between non-responders and responders are restricted to the kindled focus [103]. Rizzi et al. [46] reported that mdr1 mRNA is overexpressed in mouse hippocampus after the induction of limbic seizures. Then phenytoin was systemically administered to the mice, its brain-to-plasma ratio was 30% less than in mice not subjected to seizures thus indicating reduced drug-concentrations on brain.

Moreover, targeting Pgp by modulators can enhance the efficacy of AEDs. The co-administration of the unselective inhibitor verapamil proved to potentiate the anticonvulsant efficacy of the AED oxcarbazepine [104]. The third generation selective modulator tariquidar (TQD) increased the efficacy of phenytoin in a chronic rat model where spontaneous recurrent seizures where induced by electrical stimulation [105]. However, putative species differences in the substrate spectrum of the transporters need to be taken into account when extrapolating from rodent data to the clinical situation.

CLINICAL STUDIES OF PGP IN MTLE

Evidence for increased expression of Pgp has mainly been derived from epileptic tissues removed during epilepsy surgery from patients with pharmacoresistant epilepsy [3]. Tishler et al. [10] were the first to measure MDR1 expression in 19 patients undergoing resective epilepsy surgery, 15 of whom had mTLE receiving temporal lobeectomy for a mixture of pathologies (mostly hippocampus sclerosis). MDR1 mRNA level was found to be ten times

higher in 11 of the 19 resected samples compared with controls. In addition Pgp was detected by immunohistochemical staining in astrocytes where it is not normally present, suggesting novel expression. Following Tishler and colleagues' report multiple follow-up studies have confirmed this initial finding in patients with different types of epilepsy and different pathologies. Dombrowski et al. [106] applied cDNA array and found overexpression of MDR1, MRP2 and MRP5 in endothelial cells isolated from temporal lobe blood vessels of brain specimens from five pharmacoresistant patients undergoing temporal lobeectomy. Sisodiya et al. demonstrated both Pgp and MRP1 in astrocytic cells, but not in capillary endothelial cells, in the hippocampus in cases of hippocampal sclerosis [8]. More recently Aronica et al. [107] performed detailed immunostaining studies in brain sections from 16 patients with hippocampal sclerosis and found upregulation for Pgp and MRP2 in capillary endothelium consistent with an enhanced barrier function. MRP1 was detected in glial foot processes around blood vessels, possibly functioning as a "second line" defence mechanism. In addition, novel expression of Pgp, MRP1 and MRP2 was found in reactive astrocytes within the hippocampus in the CA1 and hilar regions. A recent post-mortem study showed Pgp overexpression in the sclerotic hippocampus of individuals with pharmacoresistant epilepsy, but not in post mortem seizure-free individuals or non-epileptogenic tissue with electrode-related injuries [108].

CLINICAL STUDIES OF PGP IN OTHER EPILEPSY SYNDROMES

Previous studies have shown that Pgp is ectopically expressed in surgically-resected specimens from patients with pharmacoresistant epilepsy with a variety of structural abnormalities; including malformations of cortical development such as focal cortical dysplasia (FCD) [109, 110]. FCD is one of the most common causes of pharmacoresistant epilepsy. Evidence for Pg expression in FCD has mainly been performed in epileptogenic human brain tissue resected during epilepsy surgery. Sisodiya et al. [111] were the first to show an overexpression of Pgp in FCD. They studied four samples of surgically resected FCD and compared the findings with normal necropsy brain tissue. They showed an overexpression of Pgp in abnormal giant dysplastic neurons, glial cells, and in perivascular distribution in epileptogenic tissue. The authors extended their work by increasing the number of patients and included 14 patients with FCD in their study [8]. Their findings confirmed their previous work demonstrating an overexpression of Pgp also in astrocytes, dysmorphic neurons and balloon cells of patients with FCD. These findings have since been confirmed in several other studies [110, 112-114]. Ak et al. included a higher number of patients than the previously reported and studied the expression of Pgp in the epileptic tissues resected surgically in 28 patients with FCD and compared the results with 10 normal necropsy brain tissues [110]. Normal brain showed no Pgp expression in neurons and astrocytes. In contrast to normal brain Pgp is intensely expressed in both neurons and reactive astrocytes in the vast majority of dysplastic tissues and endothelial cells of patients with FCD. These findings supported the 'transporter hypothesis' that overexpression of multidrug efflux transporters in

epileptogenic brain regions may lower drug concentrations and thereby reducing their antiepileptic effects, which may explain the refractoriness to AEDs in FCD. However, although increased multidrug efflux transporter expression has been demonstrated in human tissue of patients with FCD that had been resected in surgery it may not be functionally relevant. Therefore non-invasive brain imaging of multidrug efflux transporter function in pharmacoresistant epilepsy patients is a strategy to evaluate whether the overexpression of multidrug efflux transporters at the BBB as postulated in the 'transporter hypothesis' has any functional consequences that underlie pharmacoresistance in epilepsy.

NEUROIMAGING OF PGP WITH PET

Non invasive brain imaging of multidrug efflux transporter function in pharmacoresistant and seizure-free epilepsy patients is a strategy to evaluate whether the overexpression of multidrug efflux transporters at the BBB as postulated in the transporter hypothesis has any functional consequences that underlie pharmacoresistance in epilepsy. Additionally, PET tracers for multidrug efflux transporters could be useful to identify epilepsy patients with increased multidrug efflux transporter activity who will benefit from treatment with multidrug efflux transporter modulation drugs and therefore hold great promises for individualized medicine [115].

Functional neuroimaging with PET and SPECT in Epilepsy

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are imaging techniques that can study CNS function in vivo. Thereby a radionuclide is synthetically introduced into a molecule of

biological relevance and administered to a patient or animal. PET and SPECT cameras monitor the distribution of these radiotracers over time. In PET the radioisotope undergoes positron decay and emits a positron, which interacts with an electron in situ, producing a pair of annihilation photons moving in approximately opposite directions which can be detected outside the body by a PET camera [116]. In contrast with PET, the radiotracers used in SPECT emit a single photon (gamma ray) that is detected directly using collimators. By detecting photons "coincident" in time, PET provides more radiation event localization information and, thus, higher resolution images than SPECT. Therefore the great advantage of PET over SPECT is a higher sensitivity, better temporal and spatial resolution and ability to provide a quantitative measure of radioactivity in tissues.

Almost all efforts to develop multidrug efflux transporters ligands have focused on Pgp expressed at the BBB or in tumours. To date three different categories of imaging probes have been described to measure multidrug efflux transporters in vivo [117]. 1) Radiolabeled transporter substrates usually developed from drugs known to be substrates for Pgp. More recently 2) radiolabeled transporter inhibitors as well as 3) radiolabeled prodrugs.

Radiolabeled transporter substrates

[^{11}C]verapamil
[^{11}C]verapamil (VPM) is the best validated PET tracer to image Pgp function to date. Verapamil, a calcium channel blocker, has been found to be a substrate

for Pgp at low concentrations (which are used in PET) but also inhibits the Pgp transporter ATP hydrolysis (which initiates substrate extrusion) at high concentrations [36]. As it is well characterised both pharmacologically and with regards to its metabolic pathways it was thought to be a suitable candidate for a PET tracer to quantify Pgp function in vivo non-invasively. In 1996 Elsinga et al. [118] were the first to use PET to study multidrug resistance and to measure Pgp function. They developed a method using racemic (±) verapamil as a PET radiotracer and studied its tissue distribution in vivo in rats and in vitro with seizure-free and pharmacoresistant human ovarian carcinoma cell lines [118]. The racemic (±) verapamil consists of two enantiomers, the (S) - and (R)-verapamil. In vivo pharmacokinetics and pharmacodynamics of both enantiomers in animals and humans are different and the (S)-verapamil is more actively metabolised than the R-form, resulting in a 2.5 higher concentration of the (R)-enantiomer in plasma [119]. Furthermore (R)-verapamil exerts only 5-10% of the calcium channel blocking activity of the (S)-enantiomer and (S)-verapamil is ten times more potent in prolonging PR-intervals in humans [120]. As the enantiomers of verapamil have different pharmacokinetics, the quantification of racemic (^{11}C)verapamil may be difficult. For this reason Luurtsema et al. developed a synthesis of (R)-[^{11}C]verapamil and they found that the metabolism was slower than that of the racemic verapamil [121, 122]. (R)-[^{11}C]verapamil is a high-affinity substrate of Pgp and is therefore very effectively transported by Pgp at the BBB. This results in low brain uptake of this radiotracer thus making it difficult to detect regional differences in cerebral Pgp function. A possible strategy to overcome the limitation of the low brain uptake of

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Pgp substrate radiotracer is to perform PET scans after partial blockade by Pgp modulating drugs such as cyclosporine A (CsA) or tariquidar (TQD) [70]. The third-generation Pgp inhibitor TQD is safer than the non-selective CsA for use in human subjects and was shown to lack interaction with metabolism and plasma protein binding of (R)-[^{11}C]verapamil [123, 124]. Blocking Pgp with an inhibitor allows the radiotracer to enter the BBB and hence increase its uptake and signal in the brain.

Single tissue compartment model to measure Pgp function

The function of Pgp can be quantified in vivo from dynamic PET data using standard compartmental models. Pgp mediates movement of substrates, such as verapamil, from brain to plasma. Since we administer the Pgp substrate PET tracer ^{11}C-verapamil intravenously, its entry into the brain is limited by P-gp activity, and the neteffect is measured as K_1, the transport rate constant from plasma to brain; a low net influx from plasma to brain, indicated by a low K_1 value, therefore indicates high efflux from the brain and, thus, high Pgp activity. As shown in rodent models of Pgp overactivity [125], partial Pgp inhibition increases (R)-[^{11}C]verapamil K_1, but this increase is attenuated in areas of high P-glycoprotein activity, since a fixed dose of Pgp inhibitor (2 or 3 mg/kg) inhibits a lower proportion of binding sites in areas of high P-gp activity than in areas of low activity. Therefore partial Pgp inhibition should be used, because if Pgp glycoprotein were fully inhibited, brain uptake of ^{11}C-verapamil K_1 would be driven by passive diffusion only and differences in Pgp activity could not be detected.

Preclinical imaging of Pgp function with [^{11}C]verapamil

PET analysis with [^{11}C]-verapamil to assess Pgp function at the BBB within the intact CNS was first validated using *mdr1a* knockout mice and showed lower [^{11}C]-verapamil uptake in the wildtype mice compared to those in the mdr1a knockout mice [126]. In addition it was possible to prove that the reversal of Pgp function, i.e. inhibiting Pgp and enabling Pgp substrates to cross the BBB, can be monitored by PET. The unselective Pgp inhibitor CsA increased the [^{11}C]-verapamil accumulation to levels comparable in mdr1a knockout mice [126]. This concept was subsequently replicated in two further studies using the racemic [^{11}C]-verapamil and the unselective inhibitor CsA at different doses resulting in dose dependent increases of [^{11}C]verapamil by Pgp modulation after CsA [127, 128]. Furthermore Bankstahl et al. [124] performed paired (R)-[^{11}C]verapamil PET scans in a group of 7 healthy Wistar rats before and after administration of the new third-generation Pgp inhibitor TQD (15 mg/kg). After TQD administration, the distribution volume (DV = the brain-to-plasma ratio) of (R)-[^{11}C]verapamil was 12-fold higher than baseline and the influx rate constant K_1 of (R)-[^{11}C]verapamil into the brain, was about eight-fold higher after TQD hereby, demonstrating that (R)-[^{11}C]verapamil PET combined with TQD administration is a promising approach to measure Pgp function at the BBB. Recently Kuntner et al. used (R)-[^{11}C]verapamil PET in rats before and after the administration of different doses of the selective Pgp inhibitors TQD and elacridar [129]. They demonstrated that the median effective dose (the dose required to achieve 50% of the desired response in 50% of the population=ED 50) for TQD is 3.0 +/- 0.2 mg/kg. Furthermore, PET scans after 3 mg/kg TQD

resulted in regionally different enhancement of brain activity distribution, with lowest distribution volume in cerebellum and highest distribution volume in thalamus pointing to regional differences in cerebral Pgp function and expression in rat brain.

Preclinical imaging of Pgp function in pharmacoresistant epilepsy with [^{11}C]verapamil

Following the promising results of (R)-[^{11}C]verapamil in healthy Wistar rats a proof of concept study was performed in rats 48 hours after pilocarpine-induced status epilepticus (SE). Both control and post-SE rats underwent (R)-[^{11}C]verapamil PET scans after administration of tariquidar at 3mg/kg [125]. Regional PET data was analyzed and Pgp expression was independently quantified in the same brain regions using immunohistochemical staining [125]. In brain regions with increased Pgp expression (cerebellum, thalamus, hippocampus) in SE rats the influx rate constants from blood to brain, K_1, of (R)-[^{11}C]verapamil were significantly decreased relative to control animals, thereby supporting the hypothesis of regionally increased cerebral Pgp function in epilepsy.

Clinical Imaging of Pgp function with [^{11}C]verapamil

Pgp function at the BBB of healthy humans has been imaged and quantified using [^{11}C]-verapamil [130]. Recent studies have shown, however, that imaging of Pgp function at the BBB in humans may need to take into consideration a subject's age and the genetic polymorphisms of Pgp. Bartels et al. [131] studied the Pgp function using [^{11}C]-verapamil PET in 17 healthy volunteers with age

18-86. Analysis with statistical parametric mapping showed significantly decreased Pgp function in older subjects than in younger subjects in the internal capsule and corona radiata white matter and in orbitofrontal regions; thereby suggesting that Pgp function declines with increasing age. Furthermore, haplotypes of nucleotide polymorphisms at positions 1236, 2677 snf 3435 of the MDR1 gene have been shown to alter Pgp activity in vivo and to alter substrate specificity in vitro. However, imaging studies with [^{11}C]-verapamil show that pharmacokinetics were unaffected in healthy volunteers who expressed either the TTT or the CGC (wild-type) haplotype [132, 133].

The promising concept of performing (R)-[^{11}C]verapamil PET scans after blockade of Pgp was recently translated into healthy human subjects [123]. Five healthy volunteers underwent paired (R)-[^{11}C]verapamil PET scans before and after intravenous administration of tariquidar (2 mg/kg of body weight). TQD administration resulted in significant increases in K_1, +49% +/- 36% of (R)-[^{11}C]verapamil across the BBB. The data from this first human study were re-analyzed region wise using an automated atlas approach to define 43 different brain regions as well as parametric maps. No regional differences in TQD-induced Pgp inhibition were detected, suggesting that there were no regional differences in Pgp function in healthy human brain [134]. Shortly after, Eyal and co-workers confirmed the results in a study with [^{11}C]-verapamil before and during infusion of CsA (2.5 mg x kg(-1) x h(-1)) [135]. Thereby K_1 estimates were similar across gray-matter regions of the brain and the magnitude of Pgp inhibition was comparable across BBB-protected brain structures.

Clinical imaging of Pgp function in pharmacoresistant epilepsy with [^{11}C]verapamil

The use of PET to determine Pgp function in epilepsy patients has only started recently. A pilot study by Langer et al. [136] used PET and the radiotracer (R)-[^{11}C]verapamil to test for differences in Pgp activity between epileptogenic and non-epileptogenic brain regions of patients with pharmacoresistant unilateral mTLE. In this study of five subjects, there was a trend toward asymmetric uptake of (R)-[^{11}C]verapamil, favoring the temporal cortex and hippocampus ipsilateral to the main seizure focus. Parameter asymmetries were most prominent in parahippocampal and ambient gyrus, amygdala, medial anterior temporal lobe and lateral anterior temporal lobe. In contrast to temporal lobe volumes of interest asymmetries were minimal in regions not involved in epileptogenesis located outside the temporal lobe. A caveat of (R)-[^{11}C]verapamil is that the peripheral metabolism of the radiotracer is significantly faster in epilepsy patients compared to healthy controls. This is most likely caused by hepatic cytochrome P450 enzyme induction of AEDs [137]. The difficulty is that these radiometabolites which are generated from the (R)-[^{11}C]verapamil are also taken up into the brain tissue independent of Pgp function and compromise the quantitative measurement of Pgp function especially when comparing different study groups such as patient and healthy controls. Additionally, VPM is a high-affinity substrate of Pgp and is therefore very effectively transported by Pgp at the BBB. This results in low brain uptake of this radiotracer thus making it difficult to elicit and detect regional differences in cerebral Pgp function. To overcome this limitation, dynamic PET scans after

partial blockade by Pgp modulating drugs such as cyclosporine A (CsA) or tariquidar (TQD) can be undertaken [70]. Using in-vivo PET experiments with the Pgp-substrate (R)-[^{11}C]verapamil (VPM), we studied 14 pharmacoresistant mTLE patients and eight seizure-free mTLE patients due to unilateral hippocampal sclerosis (HS) as well as 13 healthy controls, testing the hypotheses that Pgp-activity is higher in pharmacoresistant than seizure-free mTLE patients and healthy controls, and that Pgp overactivity is most pronounced in the epileptic focus [138]. Despite minimising the effect of different VPM metabolism between controls and patients by only using the first ten minutes of data, we found a significant difference in VPM-K_1 globally across all analysed brain regions. VPM-K_1 values in whole brain were lower in healthy controls compared to pharmacoresistant mTLE patients but not different compared to seizure-free mTLE patients. Voxel-based SPM analysis showed that at baseline, compared to seizure-free patients, pharmacoresistant mTLE patients had significantly lower regional VPM-K_1, which corresponds to increased Pgp activity, in temporal lobe regions. In pharmacoresistant mTLE patients, VPM-K_1 correlated inversely with average monthly seizure frequency, measured at the time of the baseline PET scan (correlation for whole brain: $r =-0.651$, $p= 0.016$; hippocampus: $r =-0.604$, $p=0.029$). Because of differences in VPM metabolism between mTLE patients and healthy controls we created individual VPM-K_1 images, which were normalised for global whole brain differences in VPM-K_1 arisen from this measure being a composite of parent VPM and its metabolites. These globally normalized images were then used for further analysis to detect regional differences between healthy controls and the

two mTLE patient groups. Voxel-based comparison of globally normalised images revealed lower VPM-K_1 in pharmacoresistant mTLE patients in temporal lobe regions compared to healthy controls. Voxel-based SPM analysis before and after TQD showed significant differences in increases of VPM-K_1 between pharmacoresistant mTLE patients and healthy controls with the maximum difference in the ipsilateral hippocampus, suggesting that there is regionally specific Pgp overactivity for the epileptogenic hippocampus which is functionally relevant.

As it already has been discussed the direct evidence for increased expression of Pgp in humans at the BBB is limited to studies from post-mortem and from epileptic tissues removed during epilepsy surgery from patients with pharmacoresistant epilepsy [8, 10, 106-108], which have substantiated assumptions implicit in the transporter hypothesis that Pgp is likely to be the most important transporter in pharmacoresistant epilepsy at a structural level. However, the functional relevance of this increased expression in humans cannot be assessed ex vivo. We therefore compared the results of their VPM-PET scans with their epileptic tissues removed during epilepsy surgery [138], testing the hypotheses that Pgp overactivity demonstrated with VPM-PET correlates with Pgp expression established in surgically-resected brain tissue. Pgp immunoreactivity was observed in the blood vessels, glia, and neurons in the hippocampus and temporal cortex of the five PET-scanned pharmacoresistant mTLE patients who underwent surgery. In three pharmacoresistant mTLE patients, increases in VPM-K_1 after TQD were less pronounced, suggesting relatively higher Pgp activity, in the ipsilateral (sclerotic)

hippocampus than the ipsilateral temporal neocortex. In accordance with this observation, the same three pharmacoresistant mTLE patients also showed a higher percentage area of Pgp immunopositive labelling in the sclerotic hippocampus compared with the ipsilateral neocortex. Our in-vivo PET measurements of Pgp activity correlated with ex-vivo Pgp expression in the surgical temporal lobe specimens of those patients who had undergone surgery, in keeping with the hypothesis that there is localized Pgp overactivity in pharmacoresistant mTLE. This is the first study to date comparing in-vivo Pgp activity by using VPM-PET before and after Pgp inhibition with ex-vivo Pgp expression from pharmacoresistant mTLE patients who underwent temporal lobe resections. In particular, our study is the first study to assess whether this Pgp overexpression in pharmacoresistant mTLE is functionally relevant by combining in-vivo VPM-PET investigated Pgp activity with ex-vivo analysis of Pgp expression in surgically resected tissue. Recently Bauer et al. [139] examined seven patients with mTLE in a longitudinal study using VPM-PET before and after temporal lobe resections to assess whether postoperative changes in seizure frequency and AED load are associated with changes in Pgp function and correlated their in-vivo VPM-PET measurements of Pgp function with ex -vivo immunohistochemistry from surgical temporal lobe specimens. They only performed VPM-PET scans at baseline and did not perform PET scans after Pgp inhibition. The seven patients were followed up for a median of six years (range 4–7) after epilepsy surgery. They found that pharmacoresistant mTLE patients who became seizure-free after surgery had lower VPM-K_1 values, hence increased temporal lobe Pgp activity before surgery, increased

Pgp expression in their surgically resected hippocampal specimens, and reduced global Pgp activity postoperatively, i.e. higher VPM-K_1 values postoperatively, compared with patients who continued to have seizures postoperatively and had a poorer surgical outcome. Their results are consistent with our findings in seizure-free mTLE patients who have higher VPM-K_1 values, i.e. reduced Pgp activity, compared to pharmacoresistant mTLE patients. A recent post-mortem study showed Pgp overexpression in the sclerotic hippocampus of individuals with pharmacoresistant epilepsy, but not in post mortem tissue of seizure-free individuals or non-epileptogenic tissue with electrode-related injuries [108] indicating that seizures are necessary, but not sufficient, for increased Pgp expression. These findings suggest that (i) there is measurable, localized Pgp overactivity in pharmacoresistant mTLE which is related to seizure activity; (ii) Pgp overexpression is not seen in seizure-freedom. Pgp overactivity might thus explain why pre-treatment seizure density is one factor predicting poor response to AEDs.

Genetic polymorphism of Pgp might play a role pharmacoresistant mTLE. Only two imaging studies have been performed so far to investigate the role of Pgp and of its polymorphisms in pharmacoresistant mTLE. In a pilot study with seven patients with mTLE no apparent relationship between the ABCB1 genotype and the R-[^{11}C]verapamil efflux rate constant k_2 could be described but the sample size was small [136]. On the other hand a SPECT study in patients with epilepsy the 3435CC genotype was associated with reduced brain uptake of (99mTc)sestamibi, which was correlated with drug resistance [140].

Furthermore, phenobarbital concentration in the cerebrospinal fluid and CSF/serum phenobarbital concentration ratio were significantly lower in patients with the CC genotype than in patients with CT or TT genotypes of the ABCB1 C3435T polymorphism. Additionally, the seizure frequency was also higher in CC homozygotes [141].

[^{11}C]-N-desmethyl-loperamide

The radiotracer [^{11}C]-N-desmethyl-loperamide has the advantage that it is metabolised to a lesser extent than (R)-[^{11}C]verapamil. It has been used in rats [142] as well as monkeys [143] and the first human studies using PET with [^{11}C]-N-desmethyl-loperamide at baseline [144] and after Pgp inhibition with TQD [145] have been recently carried out. At baseline there is virtually no uptake of this radiotracer in the brain. After 2 mg/kg of TQD the brain uptake of radioactivity increased only slightly (approximately 30%). In contrast, 4 and 6 mg/kg of TQD increased brain uptake two- and four-fold, respectively. Until now, [^{11}C]-N-desmethyl-loperamide has not been used clinically to study disease in patients. On the other hand, [^{11}C]-N-desmethyl-loperamide is a high affinity Pgp substrate that virtually shows no brain uptake which makes it unsuitable to map regional differences in Pgp function at baseline.

Radiolabeled Antiepileptic drugs

It is also possible to label AEDs with a positron emitter. [^{11}C]phenobarbital and [^{11}C]phenytoin have been shown to be weak substrates for Pgp and are expected to have higher brain uptake than the high-affinity Pgp substrate [^{11}C]verapamil [79, 146]. They could therefore be better suited to assess

regional differences in Pgp function (Mairinger et al., 2011), in particular Pgp overexression. A study in eight patients with pharmacoresistant partial epilepsy and two patients without epilepsy showed that [^{11}C]phenytoin concentration ratios were lower in the visual cortex in epilepsy patients who had an average value of 1.32 (range 1.05-1.66) compared to 1.61 (1.34-1.87) in nonepileptic patients [147]. But whether phenytoin concentrations are lower within the epileptic focus was not addressed in this study [147].

Other radiolabeled Pgp substrates

Several more radiolabeled drugs and radioligands have been investigated as PET tracers for Pgp. The radioligand [^{18}F]MPPF has been developed as an alternative to short lived (^{11}C)-labelled tracers for PET studies of serotonin 5-HT$_{1A}$ receptors. In microPET studies, treatment with CsA globally increases the uptake of [^{18}F]MPPF in rat brain indicating that [^{18}F]MPPF must be a Pgp substrate [148]. So far [^{18}F]MPPF has been used in animal studies together with the third generation Pgp inhibitor TQD to study Pgp activity [149, 150]. By using [^{18}F]MPPF with TQD Bartmann et al. [150] revealed differences in Pgp function between pharmacoresistant and seizure-free epileptic rats. TQD pre-treatment increased the magnitude of [^{18}F] MPPF K$_1$ in the hippocampus by a mean of 142% in the non-responders, which significantly exceeded the 92% increase observed in the responder group. The same treatment decreased the mean magnitude of [^{18}F] MPPF k$_2$ in the hippocampus by 27% in non-responders, without comparable effects in the responder group [150]. Additionally, Bartmann et al. [150] demonstrated that the percent reduction of seizure frequency in

response to phenobarbital negatively correlated with the impact of tariquidar on the hippocampal [18F]MPPF K_1 (r = −0.5940; p < 0.05). Moreover, preliminary results obtained in a clinical study with ten mTLE patients showed that the Pgp inhibitor CsA significantly increased the [^{18}F]MPPF binding potential (the ratio of receptor density and radioligand affinity [151]) by 14% in most brain regions, regardless of their involvement in seizure generation or propagation [152]. On the other hand, a recent study using [^{18}F]MPPF PET in mice and non-human primates together with CsA [153] revealed discrepancies in the Pgp-mediated transport of [^{18}F]MPPF between mice and non-human primates. Their in vitro data indicates that [^{18}F]MPPF is not a substrate of human P-gp and that the effect of the Pgp inhibitor CsA on the brain transport of [^{18}F]MPPF in non-human primate is related to an increase in the free fraction of tracer in the plasma, concluding that it is unlikely that the kinetics of [^{18}F]MPPF brain transport and distribution are affected by Pgp activity in humans. In contrast in situ brain perfusion showed that Pgp restricted the permeability of the mouse BBB to ^{3}H-MPPF [153].

The PET radioligand [^{11}C]flumazenil which is clinically used for the assessment of GABA$_A$ receptor mediated inhibition in epilepsy and to localize epileptic foci prior to epilepsy surgery has also been suggested to be a Pgp substrate [154, 155]. [^{11}C]flumazenil was recently used to detect regional differences in Pgp functionality in five different brain regions of control and kainate-treated rats, a model for TLE, before and after TQD administration. The study showed that the GABA$_A$ receptor density (B_{max}) was reduced in kainate-treated rats, compared

with controls and that there were region-specific Pgp differences, with the hippocampus showing the highest B_{max} [156].

Furthermore, [^{11}C]colchicine [157], [^{11}C]daunorubicin (Elsinga et al., 1996), [^{11}C]carvedilol [158], [^{11}C]-GR218231 [159], [^{18}F]paclitaxel [160], various 64Cu-labeled copper complexes [161], 67Ga/68Ga radiopharmaceuticals [162], [^{11}C]TMSX, [^{11}C]MPDX, ^{11}C]donepezil [154], [^{11}C]carazolol and [^{18}F]fluorocarazolol [163] have been used to study Pgp. However, only limited in vivo data have been reported so far and it is not yet clear if these radiotracers are useful for PET studies to image Pgp function in human.

Radiolabeled transporter inhibitors
Another complementary approach to assess the Pgp system with PET is the use of radiotracers which bind to Pgp without being transported. Such radiotracers would asses the transporter distribution and give a signal increase rather than a decrease (as will Pgp substrates) in brain regions that overexpress Pgp (Löscher and Langer, 2010). Several PET radiotracers based on the Pgp inhibitors have been reported to date: [^{11}C]laniquidar, [^{11}C]MC-18, [^{11}C]quinidine [^{11}C]elacridar and [^{11}C]tariquidar [134, 164-166]. These new radiotracers have so far only been tested in animal models of naïve, transporter knock-out and epileptic rodents with rather surprising results. The cerebral uptake was lower than expected at baseline and increased several-fold rather than decreased after administration of unlabelled inhibitors [134, 164, 165]. These radioligands were administered at very low (tracer) concentrations and could behave differently than at high concentrations. It is hypothesized that the low brain

uptake, i.e. the rather substrate-like behaviour of these radiotracers, could be caused by other multidrug efflux transporters (such as BCRP) at the BBB transporting the radiotracers out of the brain [117].

NEUROIMAGING OF PGP WITH SPECT

[^{99m}Tc]sestamibi was originally developed as a K+ analog for imaging myocardial ischemia. It has been shown to be a substrate for Pgp [167]. In humans, [^{99m}TC]sestamibi was reported to image Pgp efflux transport in multidrug resistant cancers (Luker et al., 1997). However, it was found not to be an ideal substrate radioligand as it is not a selective substrate for Pgp but also for other multidrug efflux transporters (such as MRP1) (Hendrikse et al., 1998). Furthermore in contrast to PET, SPECT does not enable quantitative measurements of uptake and efflux kinetics of Pgp substrates and thus has only limited use in studying Pgp function in the BBB (Löscher and Langer, 2010).

DISCUSSION AND FUTURE WORK

Over the recent years molecular imaging techniques have helped to investigate mechanisms of pharmacoresistance in epilepsy. In particular PET has been used to evaluate the transporter hypothesis and demonstrated that Pgp is increased in pharmacoresistant epilepsy. Several open questions remain and molecular imaging techniques can help to enhance our understanding of underlying mechanisms for pharmacoresistance in epilepsy.

Using non-ivasive imaging with PET enables us to identify individual patients where pharmacoresistance is caused by Pgp overactivity and potentially individualise treatment. Moreover, comparative studies between pharmacoresistant and seizure-free epilepsy patients can enable testing for a correlation between Pgp function and the pharmacoresponse. This imaging technique can also guide patient selection for future clinical studies. In particular, PET imaging of Pgp function may allow individualized application of approaches to overcome Pgp–associated pharmacoresistance. In the future combined imaging and clinical trials of novel treatment strategies, such as Pgp inhibitors or modulators of overexpression in patients who have Pgp overactivity on VPM-PET could be employed. Targeting Pgp by modulators can enhance the efficacy of antiepileptic drugs. The compound verapamil is a substrate for Pgp at low concentrations (which is used in PET), but, like many substrates, verapamil is also an inhibitor for Pgp at high concentrations [130]. Verapamil was among the first identified inhibitors of Pgp and it may function to block Pgp–modulated efflux of AEDs in the brain, thereby raising the intracellular concentration of

AEDs [168]. The third-generation modulator TQD increased the efficacy of phenytoin in a chronic rat epilepsy model and helped to overcome pharmacoresistance to phenobarbital in chronic rat epilepsy models [169]. On the other hand, first- and second-generation inhibitors are not specific for Pgp alone and can exert additional pharmacodynamic and pharmacokinetic effects. Third-generation inhibitors are considered fairly specific. But there is recent evidence for the third-generation inhibitor TQD that it can also affect the efflux transporter BCRP/ABCG2. Moreover, long-term inhibition of this transporter needs to take into account that Pgp serves as a protective mechanism and gatekeeper in several blood tissue barriers as well as hematopoietic cells [170]. In addition to limiting access of harmful xenobiotics to sensitive tissues or cells, Pgp also accelerates extrusion of xenobiotics based on its efflux function in the liver and kidneys. Therefore, alternate approaches that leave basal transporter expression and function unaffected might offer advantages for tolerability and safety issues. Preventing seizure-associated transporter upregulation might offer an intriguing alternate approach to overcoming transporter associated pharmacoresistance [69, 171].

Of course, the ideal PET or SPECT radiotracer for studies in epilepsy patients would be a radiolabelled AED and could represent an alternative strategy for directly probing the clinical relevance in patients showing resistance to individual AEDs. Additionally, as postulated in the transporter hypothesis, the overexpression of efflux transporters restricts the entry of AEDs into the brain; thus AED concentrations in the affected areas should be lower. Even though

microdialysis for measurement of local brain AED concentrations in human epileptic brain tissue is feasible, ethical and methodological issues limit the use beyond experimental protocols [172] and not many data are available. PET experiments with a Pgp inhibitor and radiolabelled AEDs have not been done, but will important for direct assessment of brain concentration levels of AEDs in humans.

PET is non-invasive and can study the contribution of efflux transporters in pharmacoresistant epilepsy in vivo. Thereby it can be applied to various different epilepsy syndromes. The mechanisms underlying pharmacoresistance have so far mainly been addressed in focal epilepsies, primarily in TLE [3, 8]. This is partly because temporal lobectomy is the commonest epilepsy surgical procedure performed and hippocampal or mesial sclerosis is the commonest pathologic finding in pharmacoresistant epilepsy [173]. It is therefore not surprising that temporal lobe pathologies, in particular hippocampal sclerosis, are the most studied epileptogenic lesions for the upregulation of multidrug efflux transporters [3]. It remains to be determined whether overexpressed multidrug efflux transporters are underlying mechanism of pharmacoresistance exclusive in focal epilepsies or also present in other epilepsy syndromes, such as generalised epilepsy.

PET has the benefit to study the whole brain and hereby regional differences in pharmaoresigated epilepsy can be investigated beyond the seizure focus. It is currently unclear whether epilepsy, uncontrolled seizures or chronic treatment with AEDs stimulate a global rather than regional response of Pgp function.

Increased expression of Pgp in the region of the epileptic focus was found in patients with pharmacoesistant epilepsy [3]. However studies investigating Pgp overexpressions in human brain tissue have mainly been derived from "epileptic" brain tissues removed during epilepsy surgery and there is a lack of control regions from the same subject as well as "normal" controls for comparison [3]. In rat models of TLE the Pgp overexpression was restricted to the hippocampus and parahippocampal regions [67]. On the other hand in a recent PET study assessing Pgp function in a rat model of TLE there was an increase of Pgp in regions beyond the epileptogenic focus in the thalamus and cerebellum [125]. Thus in addition to examining regional differences, PET is also able to assess whether there are discrepancies in the expression and function of multidrug efflux transporters in pharmacoesistant epilepsy.

PET can also be used to compare the expression and function of multidrug efflux transporters in different groups of AED responders such as pharmacoresistant, seizure-free and remitting-relapsing epilepsy patients. PET data in humans investigating Pgp overexpression in patients with seizure-free or remitting-relapsing epilepsy are lacking. Experimental data indicates that AED responders and non-responders differ in the extend of Pgp upregulation [69]. Additionally, neurobiological mechanisms of pharmacoresistance may be different in patients who have never responded to AEDs versus those who progressed to drug-resistance after they responded initially to therapy. Furthermore, the mechanisms of reversing pharmacoresistance may differ from those generating pharmacoresistance.

As PET is non-invasive thus several scans can be given to the same patient over the course of the disease. Therefore, the progression from naïve to acute and then to chronic seizures can truly be studied. Not only could the effect of seizures which causes an upregulation of Pgp [45, 101] be investigated but also the inherent severity model of epilepsy could be studied this way. PET allows the investigators to examine the living brain before the disease is induced and over the time course of symptoms and even therapies.

Perhaps more importantly, new treatments can be evaluated in pharmacoresistant patients over time. The transporter hypothesis has gained interest in recent years, because it opens the possibility to discover potential therapeutical targets for drug resistance by either inhibiting or bypassing Pgp or other involved efflux transporters [70]. In this regards Lazarowski et al. recently suggested using add-on therapy by co-administering Pgp inhibitors, to overcome drug resistance and contribute to the effectiveness of AED treatment [174] . For example calcium channel blockers such as verapamil or nimodipine are substrates for Pgp but also competitively inhibit the efflux of other Pgp substrates such as AEDs. In paediatric refractory epilepsy cases with persistent sub therapeutic AED blood levels, nimodipine administration together with AEDs resulted in improvement of medical condition and blood levels of AEDs [174, 175]. Similarly Ianetti et al. [176] described an 11-year old boy with status epilepticus which was unresponsive to treatment. On day 37 of continues SE treatment with verapamil was started. 1.5 hour after initiation of the verapamil infusion, the patient regained consciousness was able to breathe spontaneously

and the electrical SE promptly disappeared. Ultimately, PET could serve as a predictive tool for detection of Pgp mediated drug-resistance in epilepsy. By identifying patients with increased Pgp alternate therapeutic approaches or novel AEDs as well as straightforward decisions regarding non pharmacological treatment strategies, including epilepsy surgery.

The next step in the future will be to combine imaging and clinical trials of novel treatment strategies, with Pgp inhibitors or modulators in patients who have Pgp overactivity on VPM-PET aimed at reversing drug resistance with selection of optimal patients and assessment of molecular targets. But further development of the approach needs to also consider tolerability issues specific to the different targets [69]. In particular more evaluation is needed in view of the controversial findings which AEDs are Pgp substrates and, thus, determine the penetration of which particular AEDs are affected by Pgp. Of course, evidence that some antiepileptic drugs are affected by the human Pgp isoform and that others are not substrates, needs to be considered when drawing conclusions about the future promises of any approach to modulate Pgp expression or function. Modulating Pgp might, therefore, only help to overcome resistance to selected antiepileptic drugs, but might not help to overcome multidrug resistance. More precise knowledge about substrate specificities is, therefore, crucial to guide the putative future application of respective diagnostics as well as therapeutic strategies to selected patients.

Finally, although recent studies provided the proof of concept for the transporter hypothesis and show that there is functionally relevant Pgp overactivity in

pharmacoresistant mTLE [138], the critical question remains whether it sufficient to overcome Pgp overexpression as one putative mechanism of a multifactorial problem or that other mechanisms such as intrinsic disease severity, alterations in targets, different gene variants or network alterations need to be taken into account and in the future further investigated. Given the complexity of epilepsy, it is unlikely that pharmacoresistant epilepsy is caused by a single mechanism but instead is due to several mechanisms which may even occur in the same patient. Overcoming pharmacoresistance in epilepsy represents a challenge and will necessitate a multifactorial approach and the combined efforts of basic and clinical epilepsy researchers [177].

CONCLUSION

Pharmacoresistance in epilepsy is a complex problem and several mechanisms are likely to contribute to therapeutic failure. Molecular imaging techniques in particular PET has provided evidence for the transporter hypothesis of overexpression of multidrug efflux transporters in pharmacoresistant epilepsy. Molecular imaging methods can help to further investigate mechanisms underlying pharmacoresistance in epilepsy.

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