Chapter 5.1

Summary, discussion and future perspectives
In this chapter the main findings and conclusions of this thesis and their applications for future studies are summarized and discussed.

BACKGROUND

The blood-brain barrier and P-glycoprotein
The main role of the blood-brain barrier (BBB), which is a physical as well as functional barrier, is to regulate brain homeostasis and to protect the brain from circulating agents that are potentially harmful to neuronal function. In the past, it was assumed that small, highly lipophilic molecules with a molecular weight less than 500 Da can easily cross the BBB by passive diffusion, but more recently it has been shown that many lipophilic substances only show minimal brain uptake. It has become clear that drug efflux transporters, such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) play a major role in regulating drug transfer across the BBB, including that of small lipophilic substances. Consequently, both expression and activity of these transporters mainly determine effects of toxic substances on the brain. In this thesis, the focus is on P-gp. This drug efflux transporter is situated at the luminal membrane of cerebral capillary cells, at the epithelium of the choroid plexus and at cell membranes of tumor tissue. P-gp is known for its wide variety of substrates, including anti-epileptic drugs (AEDs), cytostatic agents and also drugs that are radiolabeled and used as positron emission tomography (PET) tracers.

The role of the blood-brain barrier in drug resistance and central neurotoxicity
During the past decades many drugs have been designed to specifically target the brain (e.g. AEDs) or, on the contrary, to specifically target locations outside the central nervous system (CNS; e.g. chemotherapy for non-CNS cancer). The latter drugs are supposed not to enter the CNS as they may be harmful to neural function. To date, however, this fine tuning process of developing drugs that cross the BBB and those that are not supposed to enter the CNS is far from optimal. When CNS drugs do not cross the BBB in sufficient amounts, drug resistance may occur. In contrast, non-CNS drugs, that enter the brain, may cause central neurotoxicity. In addition, it is thought that alterations in BBB functionality (e.g. changes in drug efflux transporter function or expression) can make patients more prone to develop drug resistance or central neurotoxicity. Changes in BBB functionality in terms of overactivity or overexpression of drug efflux transporters might lead to limited net drug influx and thus to drug resistance, e.g. seen in the treatment of epilepsy. In the epilepsy field this is known as the transporter hypothesis. Similarly, BBB drug efflux transporters may also limit the uptake of molecules that are used for diagnostic purposes, such as PET tracers, in case these tracers are substrates
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of (one of) the efflux pumps. On the other hand, changes in BBB functioning in terms of reduced activity or decreased expression of drug efflux transporters might result in insufficient protection of the brain against detrimental substances, which might lead to central neurotoxicity, e.g. in treatment of systemic cancer. The overall theme of this thesis is the role of (altered) BBB functioning and, more specifically, the role of (altered) drug efflux transporter functioning in the development of drug resistance in epilepsy and central neurotoxicity of antineoplastic treatment.

**P-glycoprotein and drug resistance in epilepsy**

As will be shown later, the transporter hypothesis in epilepsy is supported by substantial evidence, i.e. P-gp overexpression and P-gp overactivity are associated with drug resistance in epilepsy. It is thought that epileptic seizures themselves are the main cause of P-gp upregulation. However, evidence for the transporter hypothesis has mainly been obtained from animal studies and from resected brain tissue obtained from drug-resistant epilepsy patients who underwent epilepsy surgery. Ideally, one would like to know the distribution and function of P-gp in epilepsy patients in vivo. This would allow for identification of drug-resistant epilepsy patients earlier in the course of their disease, so that unsuccessful treatment with AEDs that are P-gp substrates can be avoided. Furthermore, those patients can be referred to a specialized neurosurgical center for epilepsy surgery much earlier, thereby avoiding the development of more severe seizures over time, which in turn may lead to cognitive and psychosocial disorders. Last but not least, in vivo information on P-gp expression and function may be helpful in the development of alternative therapies to overcome drug resistance mediated by P-gp upregulation. PET can be an important non-invasive method to obtain information on the P-gp status in vivo.

At the start of the studies described in this thesis, only two P-gp substrate tracers were available to assess P-gp function by means of PET, namely (R)-[^11]C]verapamil and[^11]C]N-desmethyl-loperamide ([^11]C)dLop). As these ligands are P-gp substrates, they both are transported actively out of the brain, resulting in a relatively low signal in the brain, which inversely reflects P-gp function at the BBB. In case of P-gp upregulation, the signal will be reduced even further. This reduction, however, is difficult to detect due to the low signal to noise ratio. The signal to noise ratio is also low due to cerebral uptake of radiolabeled metabolites in the case of (R)-[^11]C]verapamil. Consequently, (R)-[^11]C]verapamil is not an ideal ligand for assessing P-gp upregulation. To overcome this problem of a low PET signal, baseline[^11]C)dLop scans have been compared with[^11]C)dLop scans after P-gp inhibition in the same person. To obtain information on the functional P-gp status in a patient in clinical practice, however, a single PET scan without administration of a P-gp blocker is preferred. Therefore, novel PET tracers, designed to measure P-gp expression need to be developed.
Drug resistance in epilepsy and $^{[11]}$Cflumazenil PET

Pharmacoresistance occurs in approximately 30% of all epilepsy patients. In a subset of these patients epilepsy surgery is an option. However, before resective surgery may be planned, an accurate localization of the site of seizure onset is required. An $^{[11]}$Cflumazenil PET scan is one of the modalities that can be used in these drug-resistant epilepsy patients to assist in determining the site of seizure onset in drug-resistant epilepsy patients. This is done by the assessment of changes in gamma-aminobutyric acid A (GABA$_A$) receptor density (chapter 1). However, $^{[11]}$Cflumazenil might be a P-gp substrate in mice. If $^{[11]}$Cflumazenil would also be a P-gp substrate in humans, overactivity of P-gp at the BBB due to seizures could lead to reduced cerebral $^{[11]}$Cflumazenil uptake and thus to incorrect interpretation of GABA$_A$ receptor density.

OBJECTIVES OF THIS THESIS

As stated in the introduction (chapter 1), the general theme of this thesis was the role of BBB efflux transporters as a protector of the brain and the ensuing consequences of altered functioning of these transporters. These alterations in efflux transporter functioning may lead to drug resistance or central neurotoxicity. Of all efflux transporters at the BBB, the focus was on P-gp.

More specifically, the primary objectives of the work described in this thesis were (1) to explore the effects of altered P-gp functioning at the BBB on the interpretation of $^{[11]}$Cflumazenil scans in epilepsy, and (2) to evaluate a novel P-gp tracer that would allow for the assessment of P-gp expression in vivo. The secondary objectives were (1) to present an overview of the development of central neurotoxicity as a side effect of antineoplastic treatment in cancer, including the important role of BBB drug transporters, such as P-gp, and (2) to assess the development of central neurotoxicity in high-grade glioma patients.

In this chapter, the results of the studies described in this thesis with respect to the primary objectives are summarized. Subsequently, alterations in P-gp expression and P-gp function in epilepsy are discussed, characteristics of a good P-gp substrate tracer are presented, and an evaluation of currently available P-gp expression tracers is provided. Thereafter, the key issues concerning the development of central neurotoxicity as a side effect of antineoplastic treatment in cancer are outlined, including the role of BBB drug transporters, and the main findings regarding the assessment of the development of central neurotoxicity in high-grade glioma patients are presented and discussed.
The influence of P-gp on [11C]flumazenil transport across the BBB

One of the tools to locate the site of seizure onset prior to surgery of medically refractory epilepsy patients is the assessment of alterations in GABA_\text{A} receptor density by means of [11C]flumazenil PET.\textsuperscript{25,26} However, data from \textit{ex vivo} and \textit{in vivo} studies in rodents have suggested that [11C]flumazenil is transported by P-gp.\textsuperscript{7,8} In addition, enhanced P-gp activity has been reported in medically refractory epilepsy.\textsuperscript{16,27} If [11C]flumazenil is indeed a P-gp substrate, P-gp upregulation at the BBB due to epilepsy could lead to a decrease in cerebral [11C]flumazenil uptake and consequently this may confound interpretation of GABA-ergic function. To address this issue, it was assessed whether the established PET tracer [11C]flumazenil is indeed a P-gp substrate in rodents (\textit{chapter 2.1}). This was investigated using a genetic disruption model of P-gp (in mice only) and a pharmacological inhibition model (both in mice and rats) with one of the most potent third generation P-gp blockers, namely tariquidar.\textsuperscript{28,29} It appeared that multidrug resistant 1a/1b (\textit{Mdr1a/1b}) double knock-out (dKO) mice had 71% higher cerebral [11C]flumazenil uptake than wild type (WT) mice. After administration of tariquidar, cerebral [11C]flumazenil uptake in WT mice increased by about 80%, whereas it remained the same in \textit{Mdr1a/1b} dKO mice. In rats, cerebral [11C]flumazenil uptake increased by about 60% after tariquidar administration. Tariquidar had only a small effect on plasma clearance of flumazenil. It was concluded that [¹¹C]flumazenil is indeed a P-gp substrate in rodents. Consequently, altered cerebral [¹¹C]flumazenil uptake, as observed in epilepsy, may not reflect solely GABA_\text{A}-receptor density changes, but also changes in P-gp activity.

Earlier \textit{in vitro} transport assay studies had suggested that flumazenil is not transferred by human P-gp.\textsuperscript{30,31} Clearly, these results are in contradiction to our findings in rodents. A possible explanation for this discrepancy is that \textit{in vitro} assays might be less sensitive. This is illustrated by the finding that several transport assays, such as bidirectional transport and calcein inhibition assays were not able to identify moderate P-gp substrates.\textsuperscript{30,32} Therefore, the next step was to assess \textit{in vivo} whether [11C]flumazenil is also a P-gp substrate in humans, and if so, to determine to what extent alterations in cerebral [11C]flumazenil uptake in medically refractory epilepsy patients are due to an increase in P-gp function rather than a decrease in GABA_\text{A} receptor density (\textit{chapter 2.2}). For this purpose, drug-resistant adult patients with temporal lobe epilepsy (TLE) and MRI evidence of unilateral mesial temporal sclerosis (MTS) underwent an [11C]flumazenil scan both before and approximately 80 minutes after partial P-gp blockade using 2 mg·kg\textsuperscript{-1} tariquidar. Volume of distribution (\textit{V}_T), non-displaceable binding potential (BP\textsubscript{ND}), the ratio of rate constants (\textit{K}_1/\textit{k}_2) of [11C]flumazenil transport across the BBB, and [11C]flumazenil influx (\textit{K}_i) and efflux (\textit{k}_2) across the BBB were obtained for global brain and several smaller regions. Global brain \textit{K}_1/\textit{k}_2, \textit{V}_T and \textit{k}_2 changed significantly after tariquidar administration (23%, 10% and -15% respectively), indicating that flumazenil is indeed a P-gp substrate in humans. At a regional level no left-right differences were
observed with respect to $K_1/k_2$, $V_T$ and $k_2$ responses to P-gp inhibition. Global brain $BP_{ND}$ did not alter significantly, which is in line with animal studies showing that tariquidar had no effect on kinetics of $[^{11}C]$flumazenil binding to the GABA$_A$ receptor in both naive and kainate-treated rats. Regional comparisons between ipsilateral and contralateral baseline parameters showed a substantially lower $V_T$ and $BP_{ND}$ at the hippocampus (both approximately -19%) and amygdala (both approximately -16%), but no difference in $K_1/k_2$ was observed. This suggests that regional GABA$_A$ receptor density is changed in epilepsy. It was concluded that this study provides evidence that flumazenil is a (weak) P-gp substrate in humans. Nonetheless, this does not seem to affect its clinical role as a tracer of GABA$_A$ receptors for localising the site of seizure onset.

In humans, $[^{11}C]$flumazenil transport across the BBB was altered by tariquidar with approximately 23% compared with a change of 60-80% in rodents. This could be due to the fact that only partial P-gp blockade was achieved in humans using 2 mg·kg$^{-1}$ body weight$^{23}$, whereas full P-gp blockade was accomplished in rodents using 15 mg·kg$^{-1}$ body weight tariquidar.$^{34}$ Full blockade in patients was not attempted, as it was assumed to be potentially dangerous to give higher doses of tariquidar to patients who also use AEDs.

The finding that focal alterations in GABA$_A$ receptor density were found in epilepsy is in line with previous studies.$^8,33,35$ However, the fact that no evidence for changes in P-gp function were found, either globally or focally, was not expected in these pharmacoresistant TLE patients with MRI evidence of unilateral MTS, and needs further consideration. Perhaps flumazenil is too weak a P-gp substrate to detect regional changes in P-gp function. Earlier studies on resected brain tissue of drug-resistant epilepsy patients, animal studies and in vivo $(R)$-$[^{11}C]$verapamil PET studies in humans have shown that both P-gp overexpression and P-gp overactivity are associated with drug resistance in epilepsy.$^{16,27,36}$ Unfortunately, $(R)$-$[^{11}C]$verapamil PET studies in epilepsy patients have used the micro-parameter $K_i$ as marker of P-gp activity. It is, however, questionable whether $K_i$ can accurately reflect P-gp function, as it is dependent on blood flow and day-to-day variations in cerebral blood flow do occur.$^{37}$ This may have affected measured values. Therefore, it has been proposed to use the macro-parameter $V_T$ as a surrogate marker of P-gp function, as $V_T$ is not flow dependent and test-retest variability is good.$^{38,39}$ Thus, to date direct clinical evidence for P-gp upregulation remains scarce. This implicates that more human PET studies with both P-gp substrate and P-gp expression tracers are needed to investigate this issue further in drug resistance in epilepsy.

As $[^{11}C]$flumazenil appears to be less affected by P-gp than $(R)$-$[^{11}C]$verapamil$^{40}$ and $[^{11}C]$dLOP$^{23}$, presence and severity of P-gp overactivity need to be assessed in future studies using one of those tracers. However, as mentioned earlier, both ligands are not ideal for assessing P-gp function. Therefore, new PET tracers have been developed with
the aim to determine both P-gp function (e.g. [11C]phenytoin) and P-gp expression (e.g. [11C]laniquidar, [11C]tariquidar and [11C]elacridar).

[11C]Laniquidar, a novel P-g expression tracer

Laniquidar is a P-gp inhibitor that binds to P-gp and that, when labeled with carbon-11, could potentially be used as a tracer to assess P-gp expression. In chapter 3, this novel putative P-gp expression tracer was evaluated. First, a dosimetry study in humans was performed (chapter 3.1). For this purpose, healthy subjects each underwent a series of ten whole body PET scans within a period of approximately 70 minutes. Five blood samples were obtained during each PET study. High uptake of [11C]laniquidar was seen in liver, spleen, kidneys and lung, whereas low uptake was observed in brain. The effective dose for [11C]laniquidar was 4.76 ± 0.13 for females and 3.69 ± 0.01 μSv·MBq⁻¹ for males, which is within the range measured for other carbon-11 based tracers. It was concluded that [11C]laniquidar is a safe tracer for PET imaging with a total dose of approximately 2 mSv for a brain PET/CT (computed tomography) scanning protocol.

Next, in chapter 3.2 the optimal tracer kinetic model for [11C]laniquidar was developed and reproducibility of quantitative [11C]laniquidar brain PET studies was assessed. To this end, healthy volunteers were subjected to two (test and retest) dynamic [11C]laniquidar brain scans each of 60 minutes duration. T1-weighted MRI scans were used for co-registration and volume of interest definition. Plasma input functions were measured using an online sampling device and metabolite corrections were based on 7 manual samples. Time activity curves (TACs) were analyzed using various conventional plasma input one- and two-tissue compartment models. In addition, a dual input model with parallel parent and metabolite plasma input functions was examined, because [11C]methanol is suspected to be a radiolabeled metabolite of [11C]laniquidar and is assumed to enter the brain. Radiolabeled metabolites of [11C]laniquidar appeared quickly in plasma with parent fractions of only 50 and 20% at 10 and 60 minutes post tracer injection (p.i.). [11C]Laniquidar showed a very low first pass extraction of about 2-3%. Using conventional models, the irreversible single tissue compartment (1T1K) model provided the best fits to the 60 minutes TACs. However, using the dual input model, significantly better fits were obtained. Stable K1 results were also acquired by fitting the first 5 minutes of tissue data to the 1T1K model. For both models, reproducibility of [11C]laniquidar K1 was approximately 19%. It was concluded that accurate quantification of cerebral [11C]laniquidar kinetics is impeded by its fast metabolism and, in addition, the likelihood of radiometabolites entering the brain. Best fits were acquired using a dual input model, and as an alternative, K1 could be derived from a 5 minutes scan using the 1T1K model.

Whether K1 is the ideal kinetic parameter to measure P-gp expression remains questionable, as it is flow dependent, and subject to intra-individual day-to-day cerebral
blood flow fluctuations. However, \[^{11}C\]laniquidar’s main target is situated on the capillary wall and, therefore, \(K_{I}\) is not automatically the sum of cerebral blood flow and extraction fraction. Most likely, \(K_{I}\) reflects a combination of expression of and affinity for P-gp, and possibly perfusion. In order to clarify to what extent perfusion affects \(K_{I}\), studies with combined \[^{15}O\]water and \[^{11}C\]laniquidar scans will be needed. Furthermore, at least in theory, an irreversible 1T1K model is plausible if laniquidar is indeed an irreversible inhibitor of P-gp. However, two rodent studies have shown that cerebral \[^{11}C\]laniquidar uptake increased significantly after administration of a P-gp inhibitor at pharmacological doses. In other words, in rodents, laniquidar in tracer doses appears to act as a P-gp substrate rather than as a P-gp inhibitor. If this is also true in humans, it would explain why cerebral \[^{11}C\]laniquidar uptake is so low. This issue should be clarified using P-gp blocking studies in humans, i.e. performing paired \[^{11}C\]laniquidar scans before and after predosing with a P-gp inhibitor. Other possible explanations for low cerebral \[^{11}C\]laniquidar uptake will be discussed later in this chapter.

**Altered P-gp expression and P-gp function in epilepsy: what is known?**

First of all it must be emphasized that P-gp expression and P-gp function are two different entities. P-gp expression is used to quantify P-gp density, whereas P-gp function is a quantitative parameter that describes the rate of transport of substrates by P-gp transporters. Sometimes, “P-gp function” is referred to as “P-gp activity”.

Both entities (P-gp expression and P-gp function) are used to gain knowledge on one of the putative mechanisms of drug resistance in epilepsy. The possible mechanism that is described in this thesis is based on the transporter hypothesis: inadequate access of AEDs to their targets in cerebral tissue due to overexpression or overactivity of multidrug transporters at the BBB. The specific focus of this thesis was on the most widely studied multidrug transporter P-gp. Indeed, there is evidence suggesting that changes in P-gp expression and/or function may play a role in drug resistance in epilepsy. The first piece of evidence has been derived from resected brain tissue of refractory epilepsy patients in which \(MDR1\), the gene encoding for P-gp, was overexpressed. This was confirmed in other studies and it has been shown that other transporters, such as multidrug resistance-associated protein (MRP) 1, MRP2 and major vault protein (MVP), also are overexpressed in the epileptic human brain. Furthermore, a post-mortem study showed P-gp overexpression in the sclerotic hippocampus of drug-resistant patients with TLE when compared with the contralateral hippocampus. Overexpression was also evident in animal models of epilepsy and this has been associated with reduced brain penetration of AEDs. Furthermore, various techniques (e.g. *in vitro* transport assays and *in vivo* microdialysis in rats with specific transporter inhibitors) have shown that multidrug transporters transport a variety of AEDs including carbamazepine, phenytoin, phenobarbital, lamotrigine and felbamate. However, for some
of the AEDs, such as carbamazepine, lamotrigine and phenytoin, the reported data are conflicting.\textsuperscript{32,58} Thus, more research is needed to determine which AEDs (and/or their active metabolites) are truly affected by P-gp upregulation.

Evidence exists that P-gp inhibitors can enhance the effect of AEDs.\textsuperscript{16} For example, in a rat model of spontaneous seizures, administration of tariquidar significantly increased the antiepileptic activity of phenytoin.\textsuperscript{59} Similarly, an increase in antiepileptic effects of 10-hydroxycarbazepine and oxcarbazepine was found when verapamil was used to block P-gp activity. Pharmacokinetic studies confirmed that blocking of P-gp resulted in an increase in 10-hydroxycarbazepine levels in the brain, with no concomitant changes in plasma concentrations.\textsuperscript{60,61} In addition, there are three case reports, suggesting that blocking P-gp in refractory epilepsy patients decreases seizure frequency at least temporarily.\textsuperscript{36,62,63}

Recently, two in vivo PET studies were reported on the use of the P-gp substrate tracer \((R)-[{\textsuperscript{11}C}]verapamil in humans both with and without epilepsy.\textsuperscript{36,64} In the study of Feldmann et al. lower values for the influx rate constant \((K_1)\) values in drug-resistant versus drug sensitive patients were found in ipsilateral amygdala, bilateral parahippocampus, fusiform gyrus, inferior temporal gyrus and middle temporal gyrus.\textsuperscript{36} Bauer et al. reported lower \(K_1\) values in ipsilateral amygdala and superior temporal gyrus of drug-resistant patients with TLE when compared with a reference region that is considered not to be involved in epileptic pathways (cerebellum).\textsuperscript{64} The regions with lower \(K_1\) are thought to reflect regions of P-gp upregulation. In addition, a lower increase in \((R)-[{\textsuperscript{11}C}]verapamil brain uptake after partial P-gp inhibition was found in patients with refractory TLE compared with healthy controls.\textsuperscript{36} The authors claimed that their findings support the hypothesis of P-gp upregulation due to high seizure activity as one of the putative mechanisms of drug resistance in refractory epilepsy. Furthermore, in drug-resistant patients with TLE both studies reported a correlation between pre-operative P-gp functionality and P-gp expression measured in tissue samples: the higher the P-gp function (measured as \(K_1\)) in certain temporal regions, the higher the P-gp immunoreactivity in corresponding surgically resected specimens.\textsuperscript{36,64} However, both studies should be interpreted with care as \(K_1\) was used as kinetic parameter to reflect P-gp instead of \(V_T\).

At present, the main line of thought is that P-gp upregulation in epilepsy is primarily due to epileptic seizures.\textsuperscript{11-15,27} On the other hand, there is some evidence that AEDs themselves may contribute to P-gp upregulation.\textsuperscript{11,27,65,66} However, a recent study did not find a clinically relevant induction of human BBB P-gp functionality as a result of treatment with AEDs.\textsuperscript{67}

Most knowledge of P-gp has been derived from in vitro studies, animal studies, and by examining post-mortem or surgically removed brain tissue. However, in vitro transport assay studies to assess whether a drug is a P-gp substrate are not always in line with in vivo human PET studies (chapter 2.2). It is thought that these contradictory results are
due to the lower sensitivity of in vitro assays for detecting moderate P-gp substrates.\textsuperscript{30} Furthermore, species differences have to be taken into account.\textsuperscript{68} Finally, available human tissue data mainly report on P-gp expression rather than function. Within the context of drug resistance, overall P-gp function is more important than expression. Of course, it is likely that a higher number of P-gp transporters will also result in enhanced function. Ideally, one should measure in vivo expression and function in the same subject in order to test this hypothesis. Whether there is a role for “P-gp pharmacogenetics”, i.e. \textit{ABCB1} polymorphisms, in mechanisms that lead to drug resistance in epilepsy is not clear.\textsuperscript{69,70} Studies have reported discordant results on the association between \textit{ABCB1} polymorphisms and response to AEDs.\textsuperscript{69,71-76} This discrepancy could be due to either ethnic differences or methodological problems, such as inadequate power, different definitions of drug responsiveness, and inclusion of heterogeneous groups of epilepsy syndromes.\textsuperscript{69,70}

In conclusion, there is substantial evidence suggesting that changes in P-gp expression and/or function may play a role in the development of drug resistance in epilepsy. However, in vivo evidence in humans, especially with regard to P-gp function, is scarce. Better understanding of the role of P-gp in drug resistance requires development of both improved expression and substrate tracers.

**Characteristics of a good P-gp substrate tracer**

Unfortunately, the ideal PET tracer to determine P-gp function in vivo has not been developed yet. Nevertheless, quite a lot is known about required tracer properties for PET studies of P-gp function. This is extensively summarized and discussed in a review by Syvänen \textit{et al.}.\textsuperscript{77} To summarize, an inherent problem of P-gp substrates is that they are actively transported out of the brain. Approximately 3\% of the brain consists of blood.\textsuperscript{78} Only 0.1\% of the total brain weight comprises BBB.\textsuperscript{79,80} Transport of a tracer by P-gp takes place at the capillary walls that are spread throughout the brain. The labeled molecules that are responsible for the PET signal are the ones that “bypass” P-gp and enter the brain. In other words, the PET signal mainly comes from labeled molecules in the brain parenchyma, and their number is low in the case of a good P-gp substrate tracer. Thus, to be a good P-gp tracer, the compound needs to be both a good P-gp substrate and be able to enter the brain rapidly, i.e. to be reasonably lipophilic. However, these are two contradictory characteristics.

Another essential characteristic concerns the metabolism of a PET tracer. PET measures total radioactivity in tissue, but is not able to separate the original (parent) compound from its radiolabeled metabolites. Thus an ideal tracer should not produce any labeled metabolites. As most radiotracers are lipophilic, however, they are also prone to metabolism.\textsuperscript{77} This is not a problem if (1) the radiolabeled metabolites do not cross the BBB
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at all (no tissue signal) or (2) kinetics of parent compound and radiolabeled metabolites are exactly the same.

Two strategies to increase baseline brain uptake have been proposed by Syvänen et al.\textsuperscript{81} The first one is the use of a tracer that is more lipophilic in order to increase its passive transport into the brain. A possible disadvantage of this strategy is that more lipophilic compounds could have a higher affinity for P-gp.\textsuperscript{81} The second strategy is the use of compounds such as phenytoin, which have a lower affinity for P-gp.\textsuperscript{81} Quite recently, phenytoin was labeled with carbon-11 in order to test its usefulness as a tracer of P-gp function.\textsuperscript{81} Phenytoin is a substrate of P-gp, as shown by both transcellular transport studies\textsuperscript{32} and \textit{in vivo} rat studies in naïve and epileptic rats.\textsuperscript{56,82} \textsuperscript{[11C]}Phenytoin PET studies in rats have shown that it has more favorable characteristics for measuring P-gp function than \textit{(R)-[11C]}verapamil.\textsuperscript{44} First, it has a higher initial brain uptake in rats than \textit{(R)-[11C]}verapamil, so upregulation of P-gp is easier to detect. Second, \textsuperscript{[11C]}phenytoin has optimal brain kinetics in rats, i.e. fast transport into the brain, reaching equilibrium well within the time span of a PET scan, and an optimal rate of clearance from the brain (not too slow, not too fast). Third, in rats it is metabolically more stable than \textit{(R)-[11C]}verapamil in both brain and plasma, causing less problems with labeled metabolites, potentially making it a more accurate tool for measuring P-gp function.\textsuperscript{44} Recently, the first data of dynamic \textsuperscript{[11C]}phenytoin brain PET studies in humans have been reported.\textsuperscript{83} \textsuperscript{[11C]}Phenytoin appears to have good tracer characteristics with slow metabolism (i.e. 79% of parent tracer left at 60 minutes p.i.) and little inter-subject variation. Furthermore, tracer kinetics seem favorable with a maximum cerebral uptake at approximately 20 minutes p.i.. In addition, test-retest variability was good (\textless 10%).\textsuperscript{83} The next step would be to perform \textsuperscript{[11C]}phenytoin PET scans before and after administration of a P-gp inhibitor to assess whether this tracer is sensitive to changes in P-gp function. If so, this dual scan paradigm should also be performed in drug-resistant and drug sensitive epilepsy patients to assess whether (regional) differences in VT can be found, to further evaluate the transporter hypothesis in drug resistance in epilepsy.

When a proper P-gp substrate tracer becomes available, the next step will be to gain knowledge on the relation between P-gp overactivity and P-gp overexpression to clarify to what degree P-gp is altered. To address that question, one could scan patients prior to resective surgery with a P-gp substrate tracer, and subsequently determine P-gp expression by means of immunohistochemistry of the resected tissue specimen.\textsuperscript{52}

Another option to study the role of a possibly altered P-gp mediated uptake of a drug would be to radiolabel the drug itself and perform scans before and after administration of a P-gp inhibitor.\textsuperscript{77} In that case one would not directly be informed about P-gp function, but this type of study would indicate whether the tissue drug concentrations may be vulnerable to P-gp upregulation.
Evaluation of existing P-gp expression tracers

The main purpose for developing a P-gp expression tracer is to detect global or regional P-gp overexpression in vivo. Ideally, such a PET tracer should bind selectively and with high affinity to P-gp. Several third generation P-gp inhibitors have been radiolabeled, e.g. laniquidar, tariquidar, elacridar, MC18 and MC113. Unfortunately, results have been ambiguous as a significant increase in PET brain signal was observed after P-gp inhibition. This latter observation suggests that these tracers could be, at least to some extent, P-gp substrates as well as P-gp inhibitors. Furthermore, in wild-type mice the P-gp-mediated cerebral [11C]laniquidar signal was positively affected by the mass dose of laniquidar, which also supports the notion that laniquidar in tracer dose may act as a P-gp substrate rather than as a P-gp inhibitor. In addition, it was shown that, in mice, cerebral [11C]tariquidar and [11C]elacridar concentrations were not only dependent on P-gp, but also on BCRP. In vitro transport assays and a study using a human cell line showed that both tariquidar and elacridar were both P-gp and BCRP substrates at tracer concentrations. Furthermore, P-gp/BCRP double knockout mice showed a 10-fold higher brain concentration of [11C]tariquidar than wild-type mice, whereas this was only 4-fold higher in P-gp knockout mice and 2-fold higher in BCRP knockout mice. The suggested P-gp and BCRP substrate behaviour of these tracers might complicate interpretation of their scans even further.

To the best of our knowledge only one study reported on [11C]MC18. This tracer showed a 3-fold higher initial brain uptake than other P-gp expression tracers. In addition, after the administration of unlabeled MC18, VT of [11C]MC18 decreased with approximately 30%, suggesting specific binding to its target. It remains to be investigated, however, whether this target is indeed P-gp and whether MC18 acts as a pure inhibitor.

Another disadvantage of the P-gp expression tracers mentioned above was the low cerebral PET signal at baseline. Several explanations for this low signal have been proposed. First, these radiolabeled third generation P-gp inhibitors may, at tracer doses, indeed act as a P-gp substrate, as discussed above. Second, their target is P-gp at the BBB and the BBB only constitutes 0.1% of the total brain weight, thus the actual physical space for tracer binding is very limited. Third, these compounds are highly lipophilic and therefore are highly bound to plasma proteins. For example, the free plasma concentration of [11C]tariquidar was only approximately 0.05%, i.e. 99.95% was bound to proteins. In other words, only a negligible fraction of the tracer is available for binding to P-gp at the BBB and rate of binding and uptake will then also depend on the rate of dissociation from the proteins. Therefore, it has been suggested that radiotracers with picomolar instead of nanomolar affinity for P-gp need to be developed. Fourth, it is thought that the signal is primarily due to tracer that has reached the brain tissue.
and not by tracer that is interacting with P-gp itself, which is quantitatively of much less significance.\textsuperscript{77}

In case of \textsuperscript{[11C]}laniquidar, accurate modeling in humans is hampered by its fast metabolism with only 50\% of parent compound left at 10 minutes p.i. and possibly also by metabolites entering the brain (chapter 3.2). Unfortunately, it has to be concluded that the currently available radiolabeled P-gp inhibitors are not suitable for measuring P-gp expression \textit{in vivo}.

\section*{Future research on other BBB transporters}
Not only P-gp seems to be a relevant gatekeeper at the BBB, but also BCRP and multidrug resistant-related proteins (MRPs) are thought to be involved in drug resistance in epilepsy.\textsuperscript{51,91,92} In addition to the further development of P-gp tracers, also accurate PET tracers that allow for the assessment of distribution and functioning of these other BBB drug efflux transporters are needed. For imaging BCRP and MRP, only a few possible tracers have been reported (reviewed in ref. 93) of which 6-bromo-7-\textsuperscript{[11C]}methylpurine and 6-bromo-7-(2-\textsuperscript{[18F]}fluoroethyl)purine seem to be the most promising to image MRP1 activity.\textsuperscript{94,95} Imaging MRP1 function with the latter two tracers is based on the metabolite extrusion method.\textsuperscript{96} The basic concept is that the parent tracer enters the brain by passive diffusion, then the compound is converted to a hydrophilic radiometabolite which subsequently is transported from the brain back to the blood by MRP1.\textsuperscript{96} However, the enzymatic activity of the enzyme that converts the parent tracer is susceptible to species differences. Whether this method is applicable in humans needs to be investigated. In addition, whether this promising metabolite extrusion method is also suitable for P-gp imaging is not yet clear, as P-gp is primarily located at the apical membrane of brain capillary endothelial cells\textsuperscript{97}, whereas MRP1 is mainly expressed at the basolateral membrane. It seems difficult to develop a selective BCRP tracer, mainly because BCRP and P-gp have a substantial overlap in substrate specificity.\textsuperscript{98} Carbon-11 labeled dantrolene has been developed and proposed as a (selective) candidate BCRP tracer\textsuperscript{99}, but no \textit{in vivo} studies have been reported yet.

\section*{Central neurotoxicity of anticancer treatment}
The mechanism(s) involved in the development of central neurotoxicity due to chemotherapy are largely unknown.\textsuperscript{9} Several mechanisms have been proposed, e.g. inadequate functioning of multidrug transporters at the BBB, and changes in regulating mechanisms in the brain itself, that are supposed to maintain brain homeostasis.\textsuperscript{9} Genetic profiles may affect both mechanisms and may determine a subject’s susceptibility to experience neurotoxic adverse events due to antineoplastic treatment.

In chapter 4.1 an overview of causes and consequences of central neurotoxicity in non-CNS cancer chemotherapy was presented. Methotrexate (MTX), ifosfamide and
Cytarabine are the chemotherapeutic agents that most frequently cause CNS toxicity. In rare cases 5-fluorouracil, capecitabine, busulfan, vincristine, fludarabine, cladribine, cyclophosphamide, carmustine (BCNU), procarbazine, cisplatin, carboplatin, paclitaxel, etoposide and thiotepa may cause mild to severe neurotoxic adverse events.\textsuperscript{100-105} Cognition is the most widely studied functional outcome parameter for central neurotoxicity of chemotherapy. Insight into effects of chemotherapy on cognition has been gained mainly from studies on cognitive functioning in non-CNS cancer patients who have received chemotherapy.\textsuperscript{106-108} Breast cancer patients who are treated with systemic combination chemotherapy may suffer from (temporary) cognitive dysfunction.\textsuperscript{106, 107} Some data, however, suggest that cognitive deficits should be attributed to a cancer diagnosis in general rather than to systemic treatment as cognitive deficits have been reported in 22-52\% of non-CNS cancer patients prior to chemotherapy.\textsuperscript{109-111}

Several candidate mechanisms for the development of central neurotoxicity due to chemotherapy have been described and possible contribution of genetic subtypes have been suggested.\textsuperscript{9} Polymorphisms involving regulating mechanisms are, for example, those in folate-metabolic enzymes, deoxyribonucleic acid (DNA) repair systems, cytokine activity, apolipoprotein E, and catechol-O-methyltransferase.\textsuperscript{9,112} Proposed polymorphisms in BBB transporter genes are those of \textit{MDR1} and \textit{SLCO1A2} (solute carrier organic anion family member 1A2). The \textit{MDR1} gene encodes for P-gp and the \textit{SLCO1A2} gene encodes for OATP1A2 (organic anion-transporting polypeptide 1A2), which is an influx transporter at the BBB. The exact role of pharmacogenetics in mechanisms that lead to central neurotoxicity of chemotherapy is not fully unraveled.\textsuperscript{113}

In chapter 4.2 the incidence and severity of central neurotoxicity due to standard antineoplastic treatment in patients with newly diagnosed high-grade glioma (HGG) was evaluated longitudinally. Patients may experience cognitive deficits, which may be the result of the tumor itself or of treatment related neurotoxic adverse events. In addition, radiological abnormalities, such as white matter hyperintensities (WMH) and cerebral atrophy (CA) can evolve. In this study, these functional and morphological parameters were assessed over time, and a possible correlation between them was investigated. Following resection, HGG patients were treated with chemo-irradiation for six weeks, followed by six cycles of temozolomide. This regimen is currently the standard treatment for glioblastoma multiforme patients aged up to 70 years old with a WHO performance status ≤ 2.\textsuperscript{114} Assessments were done at six time points, namely before chemo-irradiation, post-concomitantly, after the 3\textsuperscript{rd} and 6\textsuperscript{th} adjuvant cycle, and 3 and 7 months after treatment. Gradation of WMH and CA was scored on MRI. Six cognitive domain z-scores were obtained by comparing the patients’ neuropsychological functioning with that of healthy matched controls. WMH and CA developed or progressed during follow-up in 36\% and 45\% of patients, respectively, and this change was usually mild to moderate. Cognitive performance remained stable in 52\%, improved in 18\%
and deteriorated in 30% of the patients. Usually cognitive deterioration was mild, and of all patients who experienced cognitive decline 73% had tumor progression within four months after cognitive deterioration. No clear association between functional and radiological parameters was found. It was concluded that central neurotoxicity of combined modality treatment in HGG patients, measured as radiological abnormalities, was observed in approximately 40% of the patients. However, functional impact, as expressed by cognitive performance, was limited.

The addition of temozolomide to radiotherapy did not seem to affect cognitive functioning in a negative way (chapter 4.2, 115). To the best of our knowledge, no other studies have reported cognitive status in HGG patients that have received the current treatment regimen. A decrease in cognitive functioning over time in these patients seems to reflect tumor progression instead of central neurotoxicity due to chemoradiotherapy.

It is of interest to note that 28% of the patients already had WMH before chemoradiotherapy and 64% of those had progressive WMH during follow-up. Of the patients who did not have WMH before chemoradiotherapy, only 25% developed WMH during follow-up. This raises the question whether the progression of WMH in the former patients was due to the treatment. On the other hand, it is also possible that patients who already have WMH before chemoradiotherapy are more prone to develop treatment related central neurotoxicity and possibly genetic profiles are involved here (see chapter 4.1). However, the sample size was too small to investigate a relation between the latter two.

In the present study, WMH was scored according to the scoring system of Wahlund et al.116 Another interesting imaging modality to assess central neurotoxicity of chemotherapy is diffusion tensor imaging (DTI). By means of DTI, changes in white matter tracts have been observed in brain tumor patients treated with radiotherapy.117-121 One of these studies also reported that early diffusivity alterations can predict late decline in verbal recall after radiotherapy in patients with a brain tumor.121 More research is needed to evaluate whether DTI can be used as a predictive biomarker of central neurotoxicity due to chemoradiotherapy.

Research regarding underlying mechanisms of central neurotoxicity should first focus on determining BBB P-gp function in vivo in (non-CNS) cancer patients who will receive chemotherapy in combination with pre-chemotherapy and follow-up assessments of (1) the development or progression of WMH and CA as expressed on MRI (chapter 4.2), (2) white matter tract changes with the use of DTI121, (3) neuropsychological performance, (4) genetic profiles and (5) biomarkers relevant for the proposed mechanisms. In this manner, the relation between (1) P-gp function and the development of central neurotoxicity due to chemotherapy, and (2) between MDR1 genotype and P-gp functionality can be investigated. Ideally, PET scans should be repeated to get information on (alterations in) P-gp functionality status during follow-up.
CONCLUSIONS

The studies that are described in chapter 2 provide evidence that flumazenil is a (weak) P-gp substrate in rodents and in humans. Fortunately, this does not seem to affect its clinical role as a tracer of GABA_A receptors for localising the site of seizure onset. Chapter 3 contains evidence that [^{11}C]laniquidar is a safe tracer for PET imaging with a total dose of approximately 2 mSv for a brain PET- CT protocol. However, accurate quantification of cerebral [^{11}C]laniquidar kinetics is impeded by its fast metabolism and possibly by radiometabolites entering the brain. Overall it can be concluded that the currently available radiolabeled P-gp inhibitors are not suitable to determine P-gp expression in vivo. With regard to the secondary objectives of the work described in this thesis: (1) several candidate mechanisms for the development of central neurotoxicity due to systemic chemotherapy have been described and possible contributing genetic subtypes have been suggested (chapter 4.1), and (2) the key conclusion of chapter 4.2 is that the incidence and severity of central neurotoxicity due to the current standard combined modality treatment in GBM patients, measured as cognitive functioning over time, is limited.
REFERENCES


Chapter 5.1


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