Chapter 1

General introduction
and outline of the thesis
The blood-brain barrier (BBB) is a physical and functional barrier that regulates brain homeostasis, which is necessary for the stable and coordinated activity of neurones. Furthermore, the BBB serves to protect the brain from fluctuations in plasma composition, and from circulating agents such as neurotransmitters and xenobiotics capable of disturbing neuronal function. Capillary endothelial cells of the brain are closely linked with tight junctions that limit paracellular transport. These endothelial cells lack fenestrations, and about 90% of their cell surface is wrapped by astrocytic end-feet, which maintain the properties of the BBB. Previously, it has been suggested that small, highly lipophilic molecules with a molecular weight less than 400-500 Da could simply enter the CNS by passive diffusion across the BBB, but later it was demonstrated that many lipophilic substances show only negligible cerebral uptake. It has become evident that there is a second mechanism that prevents drugs and other substances from entering the CNS. This mechanism consists of so-called “drug efflux transporters” that play a major role in regulating drug entry into the CNS, including those lipophilic substances. Consequently, the expression and activity of these transporters largely determine the impact of potentially harmful substances on CNS functioning.

A large and important drug transporter family is the adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily (ABC transporters). The ABC superfamily encloses the main drug efflux pumps that can eliminate toxins, including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 4 (MRP4). The most widely studied and probably the most important pump in this family is P-gp, a large (170 kDa) glycosylated transmembrane efflux transporter. P-gp is positioned 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 and cytostatic drugs, as demonstrated by in vivo studies in P-gp deficient knock-out animals, in vitro transport assays, and several other methods.

In the past decades, numerous drugs have been developed with the aim to target the brain, e.g. anti-epileptic drugs (AEDs) in epilepsy. On the other hand, different drugs have been developed to target non-CNS diseases, e.g. antineoplastic drugs for non-CNS cancer. The latter drugs are potentially dangerous for the CNS and should stay outside the brain. To date, however, this fine tuning process of designing drugs that are supposed to enter the brain and those that are expected to stay outside the brain is far from optimal. Drugs that have been developed for targets in the brain may have difficulties actually reaching the CNS. When such a phenomenon develops, this can result in drug resistance. On the other hand, drugs that have their target(s) outside the CNS and that are not supposed to cross the BBB, may enter the CNS and thereby may cause central neurotoxicity. The theme of this thesis is the role of BBB functioning in the development
of drug resistance and central neurotoxicity. More specifically, the role of alterations in drug efflux transporter function and expression in drug resistance in epilepsy, and central neurotoxicity of anticancer treatment is assessed and discussed respectively.

**POTENTIAL MECHANISMS OF DRUG RESISTANCE AND CENTRAL NEUROTOXICITY**

As stated previously, BBB drug efflux pumps, especially P-gp, play a major role in regulating drug transport across the BBB. Alterations in BBB functioning in terms of overactivity or overexpression of efflux transporters may lead to a further restriction of drug entry into the brain and thereby can lead, or at least contribute, to drug resistance. In contrast, altered BBB functioning in terms of underactivity or decreased expression of drug efflux pumps might lead to inadequate protection of the brain against harmful substances which, subsequently, may cause neuronal dysfunction and eventually neuronal damage. This can be defined as central neurotoxicity. An overview of frequently used terms with respect to P-gp and its altered state is shown in Box 1 with Figure 1.

**Box 1**

![Diagram](image)  
*Figure 1. Overview of frequently used terms with respect to P-gp and its altered state*  
Boxes represent synonyms
It is hypothesized that upregulation of multidrug transporters limits drug concentration in the brain by transporting substrates back to the circulation and thereby, in the case of brain targeting drugs, restricting the build-up of sufficiently high concentrations of these agents in the brain\(^7\) (“transporter hypothesis”). As a result of this mechanism, the efficacy of many drugs within the CNS is greatly limited. This can be an advantage, such as in the case of antineoplastic agents for non-CNS cancer, or a disadvantage, such as in the case of drugs for the treatment of epileptic seizures, brain tumors, or other primary brain diseases. In contrast, when multidrug transporters at the BBB function insufficiently efficacy of drugs can be too high, leading to the reverse situation. It is important to investigate the function and distribution of multidrug transporters *in vivo* to be able to further clarify their role in drug resistance and central neurotoxicity. In this thesis, the focus is on the multidrug transporter P-gp. Positron emission tomography (PET) is a non-invasive imaging method (Box 2) that makes it possible to assess the function and density of P-gp by using radiolabeled P-gp substrate and P-gp binding substances, e.g. P-gp inhibitors, as PET tracers respectively. Besides changes in blood-brain barrier function, alterations of regulating mechanisms in the brain itself (e.g. changes in deoxyribonucleic acid (DNA) repair system functioning) might also make an individual more prone to develop central neurotoxicity of chemotherapy. More specific, in this thesis we will focus on pharmacogenetic aspects of brain vulnerability. This issue will be further introduced in the paragraph titled “Central neurotoxicity of anticancer treatment”.

**PET AND DRUG RESISTANCE IN EPILEPSY**

Positron emission tomography is a non-invasive imaging method that allows for measurements of various physiological and molecular processes (Box 2). In epilepsy, PET can be used for different purposes, such as: (1) assist in identifying the site of seizure onset using \(^{[11]C}\)flumazenil PET, (2) investigate drug resistance due to upregulation of P-gp using tracers based on either P-gp substrates, to assess P-gp function, or P-gp binding substances, e.g. P-gp inhibitors, to assess P-gp expression. Both will be discussed in the following sub-paragraphs.

There is convincing evidence that increased P-gp function, i.e. increased transport of P-gp substrates, and increased P-gp expression are associated with drug resistance in epilepsy, supporting the transporter hypothesis. This evidence has been derived from resected brain tissue of refractory epilepsy patients, as well as from animal studies, and from an *in vivo* positron emission tomography (PET) study with the P-gp substrate tracer \((R)\)-\(^{[11]C}\)verapamil in humans (reviewed in refs. 8,9). It is thought that P-gp upregulation in epilepsy is caused mainly by epileptic seizures.\(^{9-14}\) More limited evidence indicates that, in addition to seizures, AEDs might contribute to P-gp upregulation in the epileptic brain.\(^{9,10,15,16}\)
Drug resistance in epilepsy and $[^{11}C]$flumazenil PET

Resistance to current drug therapy is an issue for approximately 30% of all patients with epilepsy.\textsuperscript{17} Partial seizures are the most common seizure disorder in adults\textsuperscript{18}, often stemming from focal lesions.\textsuperscript{19} The most prevalent type of partial epilepsy is characterized by complex partial seizures arising from the mesial temporal lobe.\textsuperscript{20, 21} This syndrome with partial seizures is one of the most refractory types of epilepsy: 80% of these patients still have seizures despite AED treatment.\textsuperscript{20-22} Often seizures arise in areas identified by magnetic resonance imaging (MRI) and they show a very typical video-electroencephalographic (EEG) pattern both in terms of ictal rhythms and ictal semiology.\textsuperscript{23} In some of these drug-resistant patients, epilepsy surgery is a therapeutic option. Such a drastic intervention requires accurate localization of the site of seizure onset. One of the modalities that can be used to assist in determining the site of seizure onset is $[^{11}C]$flumazenil PET. This is done by the assessment of changes in gamma-aminobutyric acid A (GABA\textsubscript{A}) receptor density.\textsuperscript{24, 25} Flumazenil binds with high affinity to the benzodiazepine binding site at the $\alpha/\gamma$ subunit interface of the GABA\textsubscript{A} receptor (Figure 2).\textsuperscript{26} Flumazenil acts as a neutral antagonist of the benzodiazepine site and binding to this receptor is both selective and reversible.\textsuperscript{27} The GABA binding site is located at the $\alpha/\beta$ subunit interface (Figure 2).

Recent ex vivo data suggest that this PET radioligand is a P-gp substrate in rodents.\textsuperscript{28} If $[^{11}C]$flumazenil would also be a P-gp substrate in humans, overactivity of P-gp at the BBB due to epilepsy could lead to decreased cerebral $[^{11}C]$flumazenil uptake and thus to incorrect interpretation of changed GABA\textsubscript{A} receptor density.
Drug resistance in epilepsy and P-gp PET tracers

Positron emission tomography can be an important non-invasive imaging method for the assessment of the distribution and function of P-gp in the brain. This is of vital importance for different reasons. First of all, it must be stated that, at present, upregulation of P-gp in refractory patients can only be confirmed by examining post-mortem or surgically removed brain tissue. When it would be possible to identify refractory epilepsy patients in an early stage of their disease, prolonged ineffective treatment with AEDs could be avoided. Early referral to a specialized neurosurgical center for epilepsy surgery might prevent the development of more severe seizures over time, which are associated with cognitive and psychosocial disorders. The availability of P-gp tracers to assess distribution and function of P-gp in drug refractory epilepsy patients could also be helpful in the development of alternative therapies for such patients, i.e. the development of drugs that do not interact with P-gp or the addition of other drugs to influence P-gp function.

At the onset of work described in this thesis, only (R)-[11C]verapamil and [11C]N-desmethyl-loperamide ([11C]dLop) were available as PET tracers of P-gp function. Both agents are substrates of P-gp and therefore their concentration in normal brain parenchyma after intravenous administration is low. For both radioligands the cerebral volume of distribution (ratio of tissue over plasma concentrations at equilibrium) inversely reflects P-gp function. In case of upregulation of P-gp, it is likely that the signal in the brain will be even lower, but this is difficult to assess because of the low signal to noise ratio. The low intracerebral concentration is not the only disadvantage of (R)-[11C]verapamil. Some of its radiometabolites are thought to cross the BBB. These metabolites are likely to be P-gp substrates themselves and they are suspected to have comparable kinetics as (R)-[11C]verapamil. Therefore, (R)-[11C]verapamil is not an ideal tracer for assessing P-gp function. In contrast to (R)-[11C]verapamil, the radiometabolites of [11C]dLop are thought not to enter the brain. Nevertheless, the problem of a low PET signal remains when using a P-gp substrate as tracer. One way to overcome this problem is by comparing coupled scans in the same subject before and after P-gp inhibition. For different reasons this is not ideal, especially not for use in a clinical setting, because of the risk of adverse effects to a P-gp inhibitor, the need for monitoring during and after administration of a P-gp inhibitor, and the extra time that is involved. Therefore, there is a pressing need to develop and evaluate novel P-gp tracers that could image increased P-gp function and also P-gp expression in vivo.
CENTRAL NEUROTOXICITY OF ANTICANCER TREATMENT

Potential mechanisms in the development of central neurotoxicity
Although the cause of central neurotoxicity of antineoplastic treatment is thought to be multifactorial, the focus of this thesis is on two basic underlying endogenous mechanisms: (1) inadequate functioning of drug efflux pumps at the BBB resulting in insufficient protection of the CNS against potentially harmful effects of chemotherapy, and (2) susceptibility of the CNS to pharmacological effects of chemotherapeutic agents due to alterations in regulating mechanisms in the brain itself. The latter mechanisms comprise functional alterations in deoxyribonucleic acid (DNA) repair systems, neuronal repair systems, metabolic pathways, and cytokine activity. Little is known about differences in predisposition to develop central neurotoxicity. Genetic profiles might play an essential role in both mechanisms by determining an individual’s vulnerability to develop neurological adverse events to chemotherapy and radiotherapy. The role of BBB drug transporter- and intracerebral regulating mechanisms in the development of central neurotoxicity, and the genetics underlying these mechanisms will be further elaborated on later in this thesis.

Assessment of central neurotoxicity
Central neurotoxicity as a side effect of anti-tumor treatment can be approached from a functional as well as a radiological point of view. Cognitive functioning is the most commonly studied functional parameter for central neurotoxicity of chemotherapy. Breast cancer patients who are being treated with systemic combination chemotherapy may suffer from (temporary) cognitive dysfunction. In brain tumor patients, however, the development of cognitive dysfunction is more complex. In these patients cognitive functioning can also be influenced by the tumor itself, by side effects of radiotherapy, by side effects of AEDs, and by epileptic seizures. For that reason, insight into the effect of chemotherapy on cognition is largely acquired from studies on cognitive functioning in non-CNS cancer patients who have received chemotherapy.

The most well-known radiological indicators of both radiotherapy and chemotherapy associated central neurotoxicity are white matter hyperintensities (WMH) and cerebral atrophy (CA). Several studies have shown that WMH and CA are associated with poor cognitive functioning in patients with low-grade glioma, primary central nervous system lymphoma, and acute lymphoblastic leukemia, which supports the hypothesis that at least part of the cognitive disturbances in brain tumor patients can be attributed to central neurotoxic side effects of antineoplastic treatment.
Box 2

Basic principles of PET

PET is a tomographic imaging technique which is able to image and measure the distribution of radiolabeled compounds as a function of time. As these compounds (e.g. drugs) are administered in tracer amounts they are called (radio)tracers or (radio) ligands. PET tracers are labeled with a positron emitter and PET is based on the decay characteristics of these positron emitters. Most positron emitters have a short half-life. For example, carbon-11 has a half-life of 20.3 minutes.

Usually, a PET scan starts with the intravenous injection of a radiotracer. Following injection, the tracer is transported by the bloodstream to its target tissue, e.g. the brain. Tracer taken up by the tissue emits positrons. Positrons have the same mass as electrons, but an opposite charge. A positron travels a short distance through tissue (~1mm) before it will combine with a nearby electron and almost instantaneously the two particles annihilate (Figure 3). This effectively results in simultaneous emission of two gamma rays (photons), each with a fixed energy of 511 keV (Figure 3). These two annihilation photons travel in opposite directions and are detected by two opposing detectors of the PET scanner (Figure 3). Simultaneous pulses from two opposing detectors indicate that the annihilation took place somewhere along the line between the two detectors (line of response; LOR). This simultaneous detection of two annihilation photons is called coincidence detection. The number of coincidence events occurring between two detectors is a quantitative measure of amount of radioactivity between the detectors.52,53

To generate images of the distribution raw PET data must be reconstructed.54 This process is beyond the scope of this thesis and will not be discussed. The next step is to translate these radioactivity measurements into quantitative values of a specific

![Figure 3. Basic physics and detection principles of PET](image-url)
(patho)physiological parameter under study using appropriate tracer kinetic models.\textsuperscript{35} The parameter of interest and corresponding tracer kinetic model depend on the tracer being used. For example, glucose metabolism is measured using \(^{18}\text{F}\)FDG, for blood flow \(^{15}\text{O}\)H\(_2\)O is used, specific binding to for example GABA\(_A\) receptors can be measured using \(^{11}\text{C}\)flumazenil, and the volume of distribution of a drug (\(V_T\)) in the brain can be assessed using the radiolabeled drug as tracer (an example is \(^{11}\text{C}\)temozolomide). Figure 4 illustrates how the measured brain signal results from a combination of specific binding to a receptor (\(C(t)_{\text{S}}\)), non-specific binding to other sites in the brain (i.e. protein binding), unbound intracerebral ligand (\(C(t)_{\text{ND}}\)) and intravascular activity. Rate constants (\(K_1, k_2, k_3, k_4\)) that describe transfer of tracer between blood, non-displaceable and specific compartments can be estimated using a kinetic model.\textsuperscript{56,57} A compartment is a physiological or biochemical space or volume in which the concentration of the radiotracer is assumed to be uniformly distributed. Free and non-specific compartments

**Figure 4. Various compartmental models to quantify PET data**

A One-tissue compartment (1TC) model
B Reversible two-tissue compartment (2TC) model
C Dual input model
D Simplified reference tissue model (SRTM)

\(C(t)_P\) Concentration of parent tracer in arterial plasma (kBq·mL\(^{-1}\))
\(C(t)_{\text{tissue}}\) Total concentration in tissue compartment (kBq·mL\(^{-1}\))
\(C(t)_{\text{m}}\) Concentration of radiometabolites in arterial plasma (kBq·mL\(^{-1}\))
\(C(t)_{\text{ref}}\) Concentration in reference tissue (kBq·mL\(^{-1}\))
\(K_1\) Rate constant for transfer from arterial plasma to tissue (mL·cm\(^{-3}\)·min\(^{-1}\))
\(k_2\) Rate constants for transfer between compartments (min\(^{-1}\))
\(k_3\) en \(k_4\) Rate constants for transfer between tissue compartments (min\(^{-1}\))
are generally combined into a single non-displaceable compartment, because kinetics 
of non-specific binding are very fast. For quantification purposes, the metabolite cor-
rected arterial plasma input curve \( C(t) \) is required, which represents delivery of the 
tracer as function of time.\(^{56}\)

For neuroreceptor studies, the two-tissue compartment (2TC) model (Figure 4B) can 
be used with two separate compartments representing non-displaceable \( C(t)_{\text{ND}} \) and 
specifically bound \( C(t)_{\text{S}} \) tracer. However, if the exchange rate is fast between these 
two compartments, a simplified one-tissue compartment (1TC) model should be used 
(Figure 4A). The resulting parameter of the 1TC model is the volume of distribution \( V_T \) 
of the tracer, containing free, non-specific and specific binding, which is the ratio of the 
concentration of the tracer in tissue to that in plasma at equilibrium \( (K_{i}/k_{o}) \).\(^{56}\) A revers-
able 1TC model with a metabolite corrected plasma input function is the established 
method for analyzing \([^{13}C]\)flumazenil studies.\(^{58}\)\(^{-61}\) When the 2TC model can be used, \( V_T \) 
corresponds to \( K_{i}/k_{o}\cdot(1+ k_{3}/k_{4}) \).\(^{52}\)

Tracers may be metabolized, especially in the liver, and the resulting radiolabeled 
metabolites will enter the circulation. In case these radiolabeled metabolites enter the 
target tissue (i.e. the brain), a dual input model may be needed. Such a model accounts 
for uptake of both original tracer and its labeled metabolites (Figure 4C). However, this 
will result in added noise due to the increase in parameters that need to be fitted.

To distinguish specific from non-specific binding, under certain circumstances refer-
ce tissue models can be used. These models do not use a plasma input function, 
but rather a region within the brain that is devoid of receptors. This so called reference 
tissue provides an indirect input function. Essential for the use of a reference tissue 
model are the assumptions that (1) the levels of free and non-specific binding (i.e. 
the non-displaceable compartment) are the same in target and reference tissues\(^{62,63}\) 
and (2) that the reference tissue is not affected by the pathology under study.\(^{62,64}\) The 
outcome measure of this reference tissue model is non-displaceable binding potential 
\( \text{BP}_{\text{ND}} \). \( \text{BP}_{\text{ND}} \) is the free fraction within the non-displaceable compartment times \( B_{\text{max}} \) 
(the number of binding sites) divided by apparent \( K_{d} \) (dissociation constant from the 
receptor), which is directly proportional to ratio of density of receptors and affinity of 
the tracer for the receptor.\(^{57}\) If the affinity is constant, then differences in \( \text{BP}_{\text{ND}} \) reflect 
differences in receptor availability.\(^{56}\) For \([^{13}C]\)flumazenil, the simplified reference tissue 
model (SRTM; Figure 4D), with pons as reference tissue, is attractive for clinical single 
studies, as it is very patient friendly (no arterial sampling).\(^{59}\)
OUTLINE OF THIS THESIS

The general theme of this thesis concerned the role of BBB efflux transporters as a safeguard of the brain and the ensuing consequences of altered functioning of these transporters. These alterations may lead to drug resistance or central neurotoxicity. 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 \([^{11}\text{C}]\text{flumazenil}\) scans in epilepsy, and (2) to evaluate a novel P-gp tracer that would allow for the assessment of P-gp expression \textit{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.

First, in \textit{chapter 2.1}, it is investigated whether the established PET tracer \([^{11}\text{C}]\text{flumazenil}\) is a P-gp substrate in mice and rats. This is done by means of a genetic disruption model of P-gp (in mice only) and a pharmacological inhibition model (both in mice and in rats) with the selective P-gp inhibitor named tariquidar. In \textit{chapter 2.2} it is assessed whether \([^{11}\text{C}]\text{flumazenil}\) is a P-gp substrate in humans and, if so, to what extent changes in cerebral \([^{11}\text{C}]\text{flumazenil}\) uptake in drug-resistant patients with temporal lobe epilepsy and evidence of unilateral mesial temporal sclerosis (MTS) on MRI, are due to changes in P-gp activity rather than to changes in GABA\(_A\)-receptor density.

In \textit{chapter 3} one of the novel P-gp expression tracers, namely \([^{11}\text{C}]\text{laniquidar}\), is evaluated. Laniquidar is a P-gp inhibitor that can be labeled with carbon-11 and subsequently used for the assessment of P-gp expression. In \textit{chapter 3.1} dosimetry and biodistribution of \([^{11}\text{C}]\text{laniquidar}\) in healthy volunteers is determined. In \textit{chapter 3.2} the optimal tracer kinetic model for \([^{11}\text{C}]\text{laniquidar}\) is investigated and reproducibility of quantitative \([^{11}\text{C}]\text{laniquidar}\) brain studies is assessed.

In \textit{chapter 4} the causes and consequences of anticancer treatment with regard to central neurotoxicity of antineoplastic treatment are described. In \textit{chapter 4.1} an overview is presented of central neurotoxicity in non-CNS cancer chemotherapy and also pharmacogenetic insights into this topic are reported. In \textit{chapter 4.2} a longitudinal assessment of the incidence and severity of central neurotoxicity of standard anticancer treatment in patients with newly-diagnosed high-grade glioma is presented.

Finally, in \textit{chapter 5}, the results of the studies are summarized and discussed and suggestions for future research are proposed.
REFERENCES

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