ACS Chemical Neuroscience

Review

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Advances in PET Imaging of P-Glycoprotein Function at the Blood-Brain Barrier

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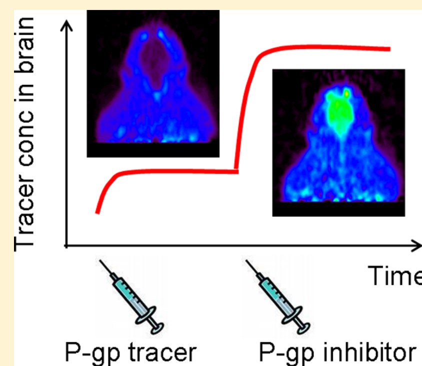
ABSTRACT: Efflux transporter P-glycoprotein (P-gp) at the blood-brain barrier (BBB) restricts substrate compounds from entering the brain and may thus contribute to pharmacoresistance observed in patient groups with refractory epilepsy and HIV. Altered P-gp function has also been implicated in neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Positron emission tomography (PET), a molecular imaging modality, has become a promising method to study the role of P-gp at the BBB. The first PET study of P-gp function was conducted in 1998, and during the past 15 years two main categories of P-gp PET tracers have been investigated: tracers that are substrates of P-gp efflux and tracers that are inhibitors of P-gp function. PET, as a noninvasive imaging technique, allows translational research. Examples of this are preclinical investigations of P-gp function before and after administering P-gp modulating drugs, investigations in various animal and disease models, and clinical investigations regarding disease and aging. The objective of the present review is to give an overview of available PET radiotracers for studies of P-gp and to discuss how such studies can be designed. Further, the review summarizes results from PET studies of P-gp function in different central nervous system disorders.



KEYWORDS: *P-Glycoprotein, CNS disease, PET, radiotracer, blood-brain barrier, efflux transporters, drug interactions*

Alteration of P-glycoprotein (ABCB1, P-gp) function at the blood-brain barrier (BBB) has been implicated in central nervous system (CNS) diseases such as epilepsy, schizophrenia, Parkinson's and Alzheimer's disease.[1−4] The main role of the BBB, which is composed of closely connected endothelial cells that separate the blood from the brain tissue, is to maintain brain homeostasis and to protect the brain from harmful substances. P-gp is an ATP-dependent 170 kDa transmembrane glycoprotein expressed at the luminal side of brain capillary endothelial cells forming the BBB where it actively transports compounds that are P-gp substrates back to the blood and thereby restricts them from reaching the brain parenchyma.[5−7] Due to this function, P-gp may act to reduce or avoid CNS side effects of some peripherally acting drugs, for example, sedation caused by antihistamines and opioid effects of antidiarrheal drug loperamide. On the other hand, P-gp may also reduce clinical effects of some drugs, for example, anti-HIV and antiepileptic drugs, and therefore at least in part contribute to pharmacoresistance.[8] The lipophilic nature of biological membranes generally allows for faster penetration of lipophilic compounds compared to hydrophilic compounds. This is especially true at the BBB since, in contrast to most other capillary membranes in the body, such as the epithelial cell layer lining the lumen of the digestive tract, the endothelial cells at the BBB have tight junctions that force molecules to penetrate through the cells rather than in between the cells. Hence, the role of P-gp in the drug delivery process is likely to be more profound at the BBB than at other membranes.

The study of P-gp function in living subjects is a challenging task, especially when the goal is to investigate the role of P-gp in human disease. One way to study P-gp in vivo is to use positron emission tomography (PET), a molecular imaging technique that can measure tissue concentrations of biomarkers (PET tracers) labeled with short-lived positron emitting isotopes. PET is a noninvasive method which does not require advanced surgical intervention and can therefore be used for investigations of P-gp function both in animals and humans. After administering the PET tracer, usually intravenously, the concentration of radioactivity residing in the area of interest is quantified using a PET scanner. This data can then be analyzed to determine the concentration of the PET tracer and interactions with binding sites as a function of time. PET imaging of P-gp function was demonstrated for the first time in 1998 with P-gp substrate verapamil labeled with ^{11}C.[9] Since then, a number of different PET tracers that image P-gp function have been described in the literature. Similar to [^{11}C]verapamil, most of them have been based on well-known P-gp substrates.[10−13] The basic idea when using labeled P-gp substrates as PET tracers is that a low brain concentration

Received: October 4, 2012
Accepted: November 25, 2012
Published: November 25, 2012

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suggests an efficient P-gp function while a high concentration indicates less efficient P-gp function. The main focus of this review is to give an overview of available PET tracers and study designs for investigations of P-gp function at the BBB and to exemplify the use of PET in related studies of different CNS diseases.

PET RADIOCHEMISTRY

A PET radiotracer is a compound labeled with a positron-emitting radioisotope, for example, ^{11}C, ^{13}N, ^{15}O, ^{18}F, or ^{68}Ga. The different chemical and physical properties of the radioisotopes make them as a group suited for a diverse range of PET applications. The physical half-life is an important factor when deciding what isotope to select, as it determines the time window during which the PET scan can take place. For example, compounds with slow kinetics (e.g., antibodies) require radioisotopes with sufficiently long half-lives, sometimes days, to enable imaging of relevant events.[14] Such large molecules can often be structurally modified without changing their pharmacokinetic properties and the choice of radioisotope then becomes less dependent on specific synthetic methods as it can be indirectly attached to the core molecular structure in numerous ways, for example, by using labeled prosthetic groups or by introducing a structure that chelates a radiometal. The study of P-gp with PET has mainly been performed with tracers with low molecular weight where even minor changes in the molecular structure can cause undesired alterations of pharmacokinetic and pharmacodynamic properties. The goal is then to design a PET tracer as close as possible to a known optimal structure. This has in turn stimulated the development of new synthesis methods for labeling of small molecules with positron emitting isotopes.[15] Many endogenous and druglike substances contain carbon (C) in positions within the molecule that are amenable for labeling with ^{11}C without modifying the chemical structure at all. This is an important reason why ^{11}C-labeled compounds often are used to study interactions of endogenous compounds and other authentic molecules in vivo. This fact is also demonstrated by the many ^{11}C-labeled P-gp PET tracers that have been investigated so far. Another possibility when labeling small molecules is direct incorporation of ^{18}F into the core of the molecule which may introduce only minor pharmacological or physiochemical changes, for example when replacing a nonacidic hydrogen atom. Another reason why ^{11}C ($t_{1/2}$ = 20.4 min) and ^{18}F ($t_{1/2}$ = 109.8 min) have become frequently used PET radioisotopes is the generally fast kinetics of small molecules that often allows the use of radionuclides with very short half-lives. The physical half-life of these two radioisotopes presents different logistical challenges during manufacturing and handling of the PET tracer, but also different opportunities when performing the PET study. Tracers labeled with ^{18}F can be distributed over a relatively large distance after manufacture, while ^{11}C-tracers require an on-site production facility. On the other hand, with ^{11}C-labeled compounds, it is possible to perform same day test−retest studies or multitracer studies in the same subject, something which is rarely possible with ^{18}F. For example, sequential PET scans in the same subject are of interest when studying P-gp function before and after administration of a P-gp inhibitor or a compound that modulates P-gp function. The half-lives of the most frequently used radionuclides in PET are given in Table 1.

Table 1. Positron Emitting Radionuclides Used With PET

radionuclide	half-life
^{15}O	2.04 min
^{13}N	9.97 min
^{11}C	20.39 min
^{68}Ga	68.3 min
^{18}F	109.8 min
^{89}Zr	78.4 h
^{124}I	100.3 h
^{64}Cu	12.7 h

PET IN BRAIN DRUG DISTRIBUTION STUDIES

PET measures the total amount of radioactive material in the tissue of interest. At its simplest, this can be quantified as the measured radioactivity, normalized to injected dose, or normalized to injected dose per body weight, given as

$$\%\text{ injected dose (\%ID)} = \frac{\text{radioactivity per tissue weight}}{\text{injected radioactivity}} \times 100 \quad (1)$$

or

$$\text{SUV} = \frac{\text{radioactivity per tissue weight}}{\text{injected radioactivity per body weight}} \quad (2)$$

where SUV is the standardized uptake value. Both of these measurements reflect the radioactive concentration at the site of measurement in relation to the amount of radioactivity injected. However, since the amount of radioactivity in the tissue is dependent on the amount supplied to it via the blood, further analysis can be performed to derive parameters that are specific for the tissue. The latter requires parallel measurements of radioactivity in whole blood or plasma during the PET scan.

The most common parameter estimated from measurements of radioactivity in tissue and plasma is the brain-to-plasma partition coefficient. The nomenclature for this parameter in PET and pharmacokinetic literature differs considerably. To clarify this, Summerfield et al. have presented a table comparing PET and pharmacokinetic nomenclature,[16] and Innis et al. have published an expert consensus for standardization of the PET nomenclature.[17] In PET, the brain-to-plasma partition coefficient is often referred to as the volume of distribution (V_T), while in pharmacokinetic studies it is called K_p.[18] This parameter describes the total concentration of a drug in tissue divided by the total concentration in plasma at steady state. V_T can be determined from PET data in several ways: by compartmental modeling,[19] by model-independent graphical analysis,[20,21] or simply by comparing steady-state concentrations in brain and plasma.[22] In addition to V_T, the net rate of drug transfer to the brain can be measured with PET if radioactivity concentrations are measured in plasma in parallel to PET scanning. This parameter is referred to as K_1 in PET literature and is comparable to the permeability surface area product PS or the net influx clearance CL_{in} used in standard pharmacokinetic literature. Other rate constants that often are estimated based on PET data are as follows: k_2, the rate constant describing the transfer of tracer from brain to plasma; k_3, the rate constant describing distribution to a second (slowly equilibrating) brain compartment; and k_4, the rate constant describing the transfer of tracer from the second brain compartment back to the first.[17,19,23] In addition to parameters

estimated based on measurements of radioactivity in blood and brain tissue, several methods exists that compare brain activity in a reference region and in a region of interest.[19,23] Application of reference tissue models allows for quantitative PET output measurement without blood sampling. However, these methods are mainly used in PET studies of receptor function, that is, when a reference region devoid of receptors exists, and are usually not utilized for studies of P-gp function.

There are a number of factors to keep in mind when conducting PET studies and especially studies using P-gp tracers that penetrate into the brain poorly. A PET scan measures the total radioactivity in the tissue and will not differentiate between signal from the intact tracer, radioactive metabolites or the radioactivity residing in the cerebrovascular volume of the brain. The vascular volume is approximately 3% of total brain volume in rats and 5% in humans.[24,25] For compounds with a low distribution into brain tissue, the vascular signal will be a considerable part of the signal measured with PET and may therefore lead to an overestimation of concentrations in brain tissue. There are different means to correct for the vascular component of the signal, and most methods are based on accurate measurement of radioactivity in whole blood. The position of the radioactive label and the molecular structure of the tracer will determine which radioactive metabolites are produced and thus contribute to the signal. Ideally, for a CNS tracer, the position of the label should be such that only relatively hydrophilic radiometabolites are produced, that is, metabolites that are unlikely to enter the lipophilic environment of the brain.[26]

Initial biodistribution studies with new drug candidates are often preclinical, and thus, the choice of preclinical species has to be considered carefully. The function of efflux transporters, in addition to systemic elimination and protein binding, may differ between species, and significant differences in brain concentrations have been reported for P-gp radiotracers across species.[27,28] PET studies involving different species have shown that even when a molecule is a P-gp substrate in rodents, it could reach relatively high brain concentrations in humans.[29,27] In fact, several radiotracers, for example, [^{11}C]flumazenil, [^{11}C](*R*)-(−)-RWAY, [^{18}F]MPPF, [^{18}F]altanserin, and [^{11}C]-GR205171, have displayed sufficient brain distributions for applications with PET, and especially [^{11}C]flumazenil and [^{18}F]altanserin were successfully used as PET CNS tracers in humans before they were known to be P-gp substrates.[30−33] Differences in brain concentrations have also been described between rodents and nonhuman primates. For example, a recent study reported that [^{18}F]MPPF was a P-gp substrate in rodents but not in nonhuman primates.[29] Concentrations of [^{18}F]MPPF were increased in monkey brain after P-gp inhibition, but this was due to an increase in the free fraction of [^{18}F]MPPF in plasma, probably caused by the P-gp inhibitor Cyclosporine A. The same observation has previously been reported also for [^{11}C](*R*)-(−)-RWAY.[34,35] Hence, these two examples illustrate the importance of measuring plasma concentrations including free fractions of radiotracers when quantifying their brain distribution.

■ PET P-GP TRACERS

There are mainly two types of P-gp PET tracers described and developed; tracers that are P-gp substrates and tracers that are P-gp inhibitors.

[^{11}C]Verapamil. The by far most used PET tracers for assessing P-gp function in vivo are enantiomerically pure (*R*)-[^{11}C]verapamil[36] and racemic [^{11}C]verapamil.[37] Verapamil is a reasonably specific P-gp substrate, although some interaction with BCRP (ABCG2, breast cancer resistance protein) has also been reported.[38] The increase in brain concentrations of [^{11}C]verapamil/(*R*)-[^{11}C]verapamil in rat after complete P-gp inhibition is 10−15-fold.[39−44] PET images using [^{11}C]-verapamil before and after P-gp inhibition are shown in Figure 1. Verapamil is also an inhibitor of P-gp, but, at the tracer doses

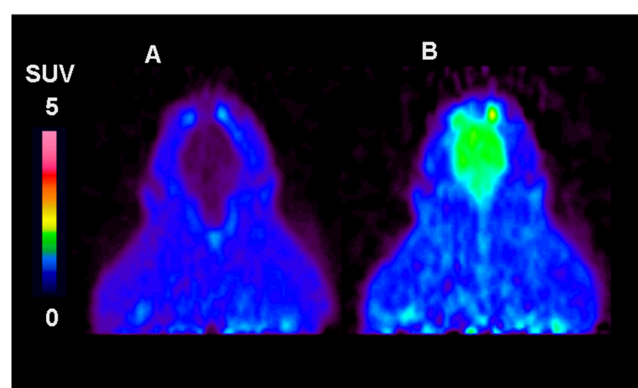

Figure 1. Average [^{11}C]verapamil PET images before (A) and after (B) intervention with P-gp inhibitor Cyclosporine A 25 mg/kg. Bright colors indicate high brain concentrations, expressed as SUV, of [^{11}C]verapamil.

used in PET, it has no inhibitor effect. In fact, its LD_{50} value in rat is so low that in vivo inhibition of P-gp with verapamil is impossible; the rats die at doses far below those required to block P-gp.[45] Although (*R*)-[^{11}C]verapamil is currently the most commonly used P-gp PET radiotracer, it is by no means ideal, mainly because of its low baseline signal and the formation of radiolabeled metabolites that likely contribute to the PET signal. One of the metabolites generated from verapamil, D617, has also been described as a P-gp substrate.[46] Not surprisingly [^{11}C]D617 is also one of the main metabolites of [^{11}C]verapamil observed when performing in vivo studies using PET (Figure 2). Verbeek et al. synthesized [^{11}C]D617 and investigated if the metabolite could be used as a tracer.[47] It was hypothesized that D617 would reach a higher brain concentration and thus give a higher PET signal, compared to [^{11}C]verapamil, since D617 is a weaker P-gp substrate. In addition, less metabolites would be produced that could confound the signal. The study showed that [^{11}C]D617 was a P-gp substrate, but brain concentrations increased only 2.4-fold compared to the 12-fold increase observed for (*R*)-[^{11}C]-verapamil[42,43] after inhibition with i.v. administered 15 mg/kg P-gp inhibitor tariquidar. This indicated either that [^{11}C]D617 is a much weaker P-gp substrate than (*R*)-[^{11}C]verapamil or that [^{11}C]D617 was distributed passively to the brain at a higher degree than (*R*)-[^{11}C]verapamil. The later is less likely as [^{11}C]D617 is less lipophilic than (*R*)-[^{11}C]verapamil and passive distribution is strongly correlated with lipophilicity. A third possible explanation to the relatively modest increase in brain concentrations of [^{11}C]D617 after P-gp inhibition could be that [^{11}C]D617 interacts with other efflux transporters in addition to P-gp, for example, BCRP.

Several studies have pursued various strategies to correct for metabolite radioactivity in (*R*)-[^{11}C]verapamil PET studies. Lubberink et al. analyzed human (*R*)-[^{11}C]verapamil data and concluded that lipophilic metabolites, which could potentially

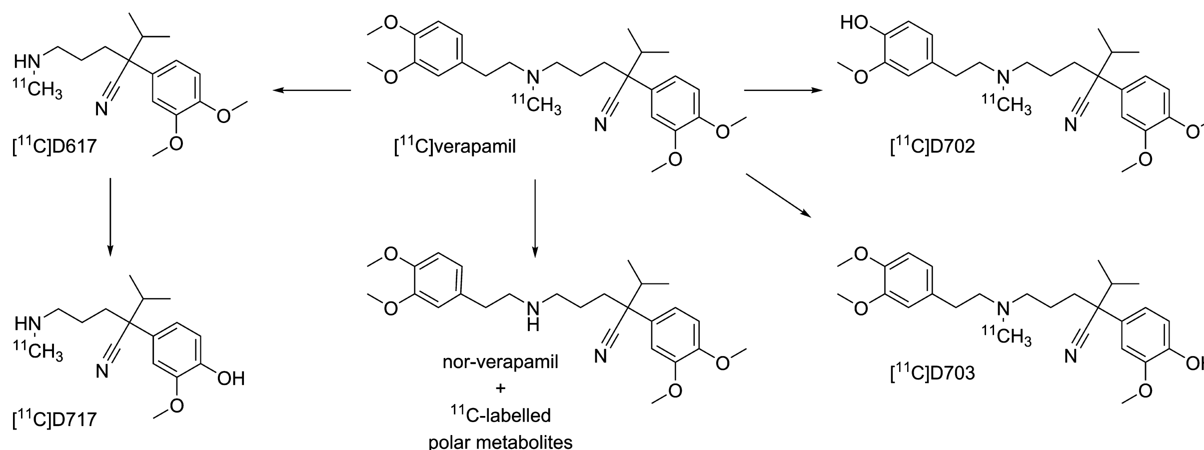

Figure 2. Metabolism of [^{11}C]verapamil produces several labeled metabolites that can confound the PET signal. To avoid the mix of several different labeled molecules, one of the main metabolites, [^{11}C]D617, has been synthesized and evaluated as a PET tracer and appeared to be a substrate with less affinity for P-gp compared to [^{11}C]verapamil.

enter the brain, can be assumed to diffuse and to be transported into the brain to the same extent as the parent compound (*R*)-[^{11}C]verapamil.[49] This appeared justified based on the results obtained with [^{11}C]D617 which showed that baseline concentrations of [^{11}C]D617 were similar to that of (*R*)-[^{11}C]verapamil,[47] and hence, it does not matter if the signal originates from (*R*)-[^{11}C]verapamil or from the labeled metabolite [^{11}C]D617, since their kinetics at baseline appear to be similar. However, since D617 displayed a lower increase in brain concentrations after P-gp inhibition than (*R*)-[^{11}C]verapamil, the two substances cannot be lumped together when P-gp function is altered. Muzi et al. suggested that data from the first 10 min of the scan would be used to estimate K_1 (transport rate of (*R*)-[^{11}C]verapamil into the brain) and since the contribution of labeled metabolites would be negligible during these early time points, the estimated K_1 would truly correspond to (*R*)-[^{11}C]verapamil transport (and not to labeled metabolites).[50] However, there is an ongoing debate whether P-gp modulation produces changes in K_1 or k_2 or in both K_1 and k_2.[39,51] Thus, most studies use V_T as the main outcome parameter, as changes in V_T incorporate changes in both K_1 and k_2 as V_T is calculated as K_1/k_2 and K_1/k_2 $(1 + k_3/k_4)$ for a one- and two-tissue compartment model, respectively.[19,23] A schematic image of P-gp and how it transports its substrates across the BBB is shown in Figure 3.

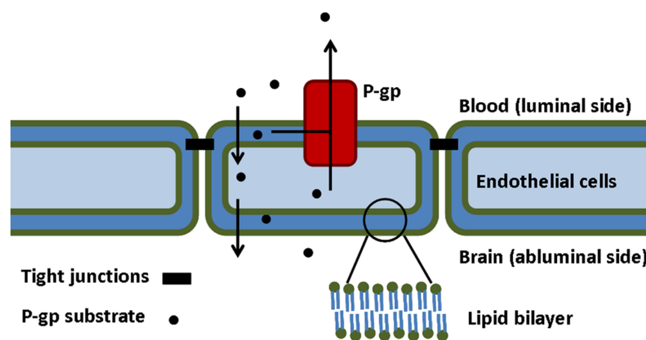

Figure 3. Drug molecule passage between the endothelial cells of the BBB is very limited due to tight junctions between cells. Thus, molecules have to pass the lipid bilayers of the BBB by passive transport. P-gp is expressed at the luminal side of the BBB, and it is today debated whether P-gp solely picks its substrates from the luminal bilayer or from the endothelial cells of the BBB as well.

[^{11}C]Loperamide and [^{11}C]Desmethyl-Loperamide. Loperamide, an over-the-counter drug acting on gut opiate receptors and used for treatment of acute diarrhea, is like verapamil a well-known P-gp substrate and has therefore also been evaluated as a substrate tracer for imaging P-gp function. It was discovered already in the first study in mice and monkeys that radiometabolites of [^{11}C]loperamide entered the brain when P-gp was absent or inhibited and thus confounded the signal from [^{11}C]loperamide.[52] The main metabolite was identified as [^{11}C]desmethyl-loperamide ([^{11}C]dLop) and was found to be a strong substrate for P-gp. Therefore, in the development of a new P-gp tracer, the focus was shifted from [^{11}C]loperamide itself to its metabolite[53] and [^{11}C]dLop was subsequently also investigated in humans.[54,55] Further, a combined in vitro and in vivo study showed that [^{11}C]dLop was selective for P-glycoprotein and did not interact with BCRP or Mrp1 at the blood-brain barrier.[56] In monkey, the increase in brain concentration after complete P-gp inhibition was 7-fold,[53] and although somewhat lower compared to (*R*)-[^{11}C]-verapamil, this should still be enough for detecting a down-regulation of P-gp function. Further, [^{11}C]dLop has been shown to be trapped within acidic lyzosomes, and this provides for an easily measurable nonreversible signal.[57] The main problem with [^{11}C]dLop is the very low baseline concentration which probably makes it unfeasible to detect an upregulation in P-gp function. Furthermore, a baseline signal that is very noisy may introduce uncertainty when comparing concentrations before and after P-gp inhibition, that is, the ratio between the brain concentration after inhibition and the concentration before inhibition.

Other P-gp Substrate Tracers. In addition to [^{11}C]-verapamil, [^{11}C]D617, [^{11}C]loperamide, and [^{11}C]dLop, a number of other radiolabeled compounds have been investigated or proposed as possible radiotracers for studies of P-gp function (Figure 4). The first PET tracer used as a P-gp function marker was [^{11}C]colchicine.[58] The tracer was not aimed for a CNS application but for distinguishing P-gp negative and positive tumors. Later studies showed that there was a 2-fold difference between positive and negative tumors, and it is therefore likely that this tracer would not be able to detect small changes in P-gp function at the BBB. [^{18}F]-Paclitaxel, also a known P-gp substrate, showed very low brain concentrations at baseline and almost no difference in brain

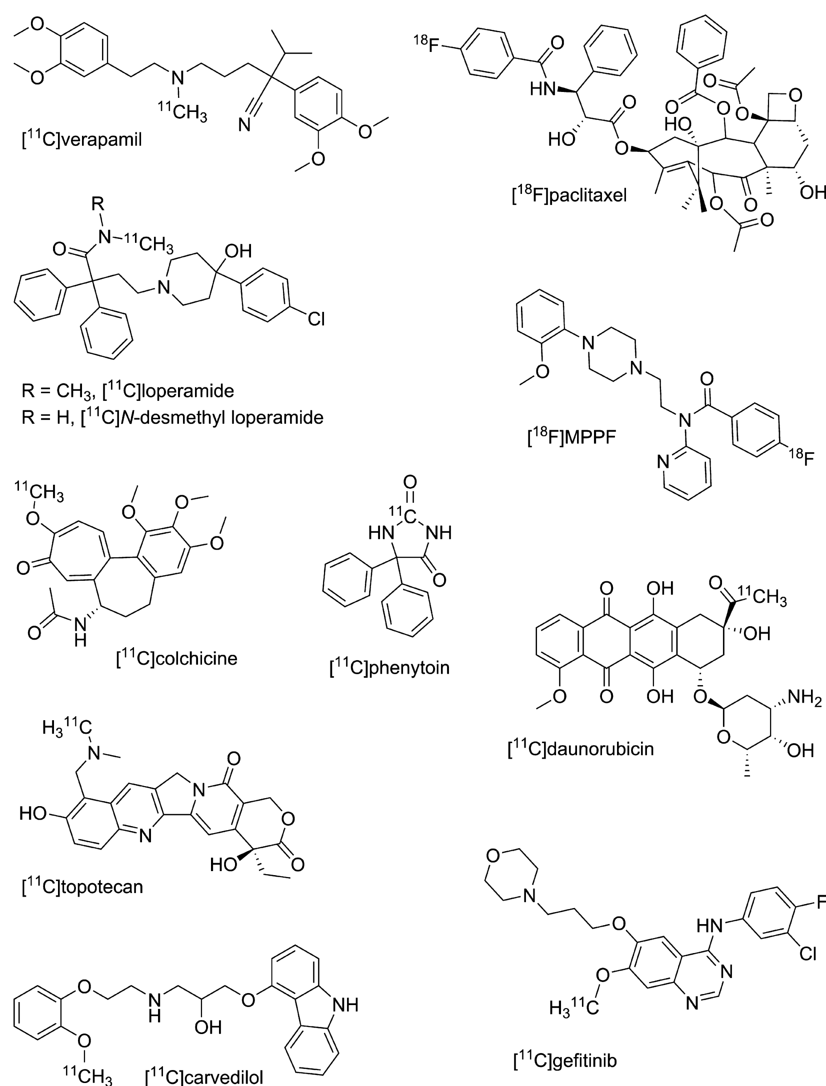

Figure 4. Structures of PET tracers that are P-gp substrates.

concentrations before and after P-gp inhibition.[59] However, the post P-gp inhibition scan was obtained 1 h after administration of 2 mg/kg tariquidar, and thus, the tariquidar dose might have been too low or the time between tariquidar dosing and PET might have been too long for actually achieving P-gp inhibition at the BBB during the PET scan. The P-gp inhibition is transient, and [^{11}C]verapamil studies have shown that the effect of low doses of P-gp inhibitors has disappeared at 1 h post dosing.[40,51] Other evaluated tracers include [^{11}C]carazolol, [^{18}F]fluorocarazolol,[60] and [^{11}C]carvedilol,[61] but also here the brain concentration differences between before and after P-gp inhibition were found to be moderate. [^{11}C]Daunorubicin gave a 16-fold concentration difference when imaging P-gp positive and negative tumor cells (human small cell lung carcinoma cell line GLC4 and GLC4/P-gp), but the brain concentrations before and after P-gp inhibition were the same and hence the tracer was not further investigated as a marker for P-gp function at the BBB.[62] Antiviral drug and P-gp substrate oseltamivir, also labeled with ^{11}C,[63,64] has been suggested as a possible PET tracer for imaging of P-gp function, but no studies using [^{11}C]oseltamivir together with a P-gp inhibitor have yet been published. Dopamine D3 receptor antagonist [^{11}C]GR218231 showed a 12-fold increase in brain uptake after complete P-gp inhibition in rats [65] but has not been further evaluated as a P-gp tracer, perhaps since it also is a receptor ligand and that this could confound studies on P-gp function. Several other receptor ligands have been shown to be P-gp substrates; brain concentrations of GABA$_A$ receptor ligand [^{11}C]flumazenil were increased 1.6- to 1.8-fold in rats and mice at complete P-gp inhibition,[66] NK$_1$ receptor antagonist [^{11}C]GR205171 3.5-fold in rats at complete P-gp inhibition,[27] 5-HT$_{1A}$ receptor antagonist [^{11}C](*R*)-(−)-RWAY 5-fold in rats at complete P-gp inhibition inhibition,[35] 5-HT$_{1A}$ receptor antagonist [^{18}F]MPPF 1.5- to 2-fold in rats and mice at complete P-gp inhibition,[29,67,68] and 5-HT$_{2A}$ receptor antagonist [^{18}F]-altanserin 2.3-fold in rats at complete P-gp inhibition.[27] In addition to these receptor ligands, other drugs have been investigated; antiepileptic drug [^{11}C]phenytoin showed a 1.5-fold increase in brain concentrations in rats after P-gp inhibition,[69] anticancer drug [^{11}C]topotecan (also a BCRP substrate) a 2-fold increase in P-gp deficient mice compared to controls,[70] and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor [^{11}C]gefitinib (also a BCRP substrate) a 4-fold increase in rats and mice[71] after complete P-gp inhibition.

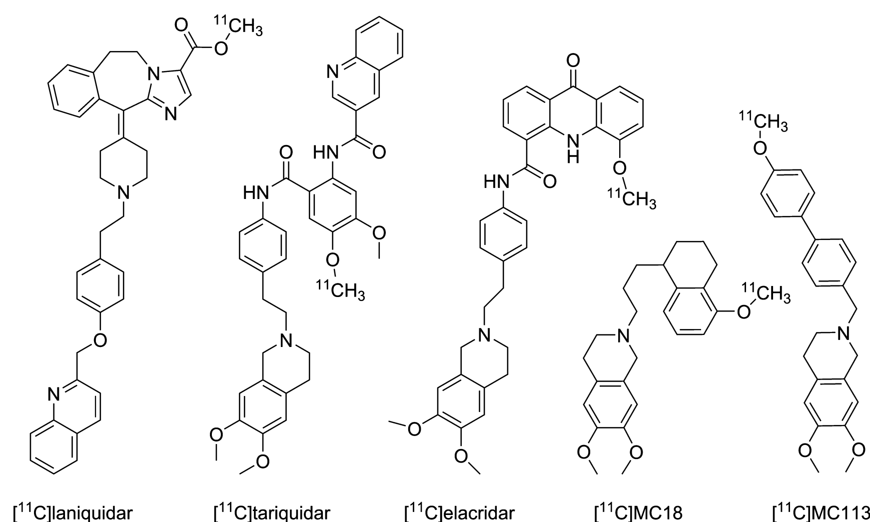

Figure 5. Structures of P-gp inhibitors that have been investigated as PET tracers.

Considerations When Using P-gp Substrate Tracers. The signal when using P-gp substrates as PET tracers arises from the fractions that escape P-gp and are partitioned in the brain tissue. Thus, for a substrate to be a good P-gp tracer, it needs to be reasonably well partitioned (by nonspecific binding) in brain tissue. A too low baseline signal is an inherent problem when P-gp substrates are used as PET tracers, and although they are able to detect a downregulation of P-gp function, it is unlikely that a small to moderate upregulation in P-gp function, which would lead to a further decrease of the PET signal compared to baseline, would be detectable with the two main P-gp tracers in use today, (*R*)-[^{11}C]verapamil and [^{11}C]dLop. To solve the problem of a weak signal, it has been suggested that (*R*)-[^{11}C]verapamil could be given with a medium dose of a specific P-gp inhibitor, such as tariquidar, to increase the baseline signal and thus create larger differences in brain concentrations of the tracer between healthy and diseased brains.[72] The drawback of this sophisticated protocol is that the concentrations of the inhibitor have to be very tightly controlled, since a small change in the concentration of the inhibitor close to the IC_{50} can result in a large change in P-gp function, thus masking any differences that may have been caused by the disease condition itself. Interindividual variability in affinity for and response to the substrate or inhibitor may also complicate the outcome when using a medium dose of a P-gp inhibitor. Simulation studies are encouraged to investigate these issues. There are also some ethical and toxicological considerations regarding this protocol, since P-gp inhibitors could give rise to side effects caused by changes in the permeability of the BBB. Another strategy to overcome the low baseline signal could be to develop a radiotracer with a higher baseline brain distribution. One way of achieving this would be by increasing the passive permeability of the tracer by making it more lipophilic, since the extent of brain uptake is dependent on both passive and active transport.[73,74] However, increased lipophilicity could also increase the P-gp affinity. In general, the currently available PET tracers are already rather lipophilic. Another way of increasing the baseline brain distribution would be to use a substrate with a lower avidity for P-gp. This strategy appears possible, and clinical studies with, for example, [^{11}C]phenytoin are being planned.[69] Okamura et al. demonstrated with Mrp1 substrate 6-bromo-7-[^{11}C]methylpurine that yet another strategy to increase the brain PET signal when imaging efflux at the BBB may be to use a tracer that readily distributes passively into the brain where it is converted to a efflux transporter substrate and thus subsequently transported out from the brain.[75]

P-gp Inhibitor Tracers. The difficulties associated with detecting upregulation of P-gp function when using PET tracers that are P-gp substrates could potentially be circumvented by using a tracer that is a pure inhibitor of P-gp.[76,77] The reasoning is that, with a P-gp inhibitor, the PET signal would increase if P-gp expression was upregulated, since this would correlate to an increase of binding sites. In other words, give the opposite response to an upregulated P-gp function compared to tracers that are P-gp substrates. A few attempts have been made to develop PET tracers based on compounds that are described as pure inhibitors, that is, compounds that have no substrate properties, for example, tariquidar, elacridar, and laniquidar (Figure 5). The first studies with these tracers gave disappointing results, as the tracers undoubtedly behaved like P-gp substrates rather than inhibitors. The increase in brain concentration after complete P-gp inhibition in rat was 8-fold for [^{11}C]laniquidar,[77] 4-fold for [^{11}C]tariquidar,[78] and 5-fold for [^{11}C]elacridar;[76] that is, the changes were of the same magnitude as observed with (*R*)-[^{11}C]verapamil. Two structural analogues of tariquidar, MC18 and MC113 (Figure 5), both labeled with ^{11}C, have also been explored as P-gp inhibitor tracers.[79,80] [^{11}C]MC18 was not studied post P-gp inhibition but showed a small decrease in V_T after pretreatment with unlabled MC18. This mainly showed that the binding to its target was specific. [^{11}C]MC113 displayed modest increased brain concentrations after P-gp inhibition and did not show decreased brain concentrations in P-gp knockout mice (mdr1a/b$^{(-,-)}$) compared to wild type mice as would be expected of true P-gp inhibitor tracers.

Two research groups demonstrated almost simultaneously that [^{11}C]tariquidar brain concentrations were in addition to P-gp also dependent on the efflux transporter BCRP.[78,81] Both groups showed that the brain concentration of [^{11}C]tariquidar was increased >10-fold in P-gp/BCRP knockout mice compared to controls. This can be compared to the 4-fold increase in P-gp knockouts and 2-fold in only BCRP knockouts. Kannan et al. further showed that tariquidar was a BCRP substrate also in a human cell line,[82] thus indicating that the results obtained in vivo in mice are likely to also represent the

situation in humans. Clearly, [^{11}C]laniquidar, [^{11}C]tariquidar, and [^{11}C]elacridar were not suitable for detecting P-gp upregulation at the BBB. However, a subsequent study using [^{11}C]tariquidar in tumor bearing mice showed more promising results.[83] Murine mammary carcinoma cells (EMT6) were continuously exposed to doxorubicin to generate a P-gp overexpressing, doxorubicin-resistant cell line (EMT6AR1.0 cells). Both cell lines were subcutaneously injected into female athymic nude mice which were scanned 1 week later with (*R*)-[^{11}C]verapamil, [^{11}C]tariquidar, or [^{11}C]elacridar before and after complete P-gp inhibition with tariquidar. In line with the previous CNS studies, concentrations of all three compounds increased after P-gp inhibition. However, at baseline, the PET signal obtained with [^{11}C]tariquidar and [^{11}C]elacridar in P-gp overexpressing tumors was higher than in non-overexpressing tumors. The opposite was found for (*R*)-[^{11}C]verapamil. This indicates that elacridar and tariquidar also have nonsubstrate properties, that is, that they bind to P-gp and that a higher density of P-gp will lead to a higher PET signal. It is likely that the P-gp density at the BBB is so low that the signal originating from direct interaction with P-gp is masked by the signal coming from tracer that has reached the brain tissue or that BCRP and P-gp efflux hinder the tracers from binding to P-gp. Both [^{11}C]laniquidar (LogP = 6.9)and [^{11}C]tariquidar (LogP = 6.1) are highly lipophilic molecules and are therefore, especially after P-gp inhibition, likely to be well partitioned into brain tissue. However, tumors do not provide the same type of lipophilic environment, and in combination with a very high density of P-gp it could be possible to observe the signal from tracer molecules interacting with P-gp. Similar to the results obtained with [^{11}C]tariquidar, also [^{11}C]laniquidar displayed a bivalent character in a study that compared administration of [^{11}C]laniquidar with or without a coinjection with a pharmacological dose of isotopically unmodified laniquidar.[84] [^{11}C]laniquidar brain concentrations were higher when administered with the coinjection which indicated that laniquidar acted as a substrate at low doses and as an inhibitor of P-gp transport at higher doses. In conclusion, the present P-gp inhibitor tracers may be of use for detecting an upregulation of P-gp expression in tumors, but will most likely not be useful for CNS applications as inhibitor tracers. However, (*R*)-[^{11}C]verapamil is not an ideal substrate tracer due to its low baseline signal and the generation of lipophilic metabolites that may be taken up in brain tissue and thus confound the signal. Thus, [^{11}C]laniquidar, [^{11}C]tariquidar, or [^{11}C]elacridar that initially were developed as inhibitor tracers could perhaps be used as substrate tracers if their metabolism and pharmacokinetics appear to be preferable compared to (*R*)-[^{11}C]verapamil.

Considerations When Using P-gp Inhibitor Tracers. Interactions between the drug and P-gp take place in the BBB, which constitutes 0.1% of the brain weight.[85,86] In comparison with PET receptor binding studies where the target tends to be spatially distributed, P-gp is more evenly spaced over the whole brain volume. This means that even if a true P-gp inhibitor tracer with no substrate characteristics would be available, it would still be difficult to use it for detecting P-gp expression as the signal must be differentiated from tracer residing in the cerebral blood fraction and from tracer that is partitioned into the brain by passive diffusion. Yet another important point with regard to the analysis of PET data obtained from studies using P-gp inhibitors as tracers is that these studies would, in a strict sense, measure P-gp expression and not function. It is important to keep in mind that it is not certain that P-gp function and expression are changing linearly, and when trying to predict drug uptake into the brain it is likely that function is more relevant than expression.

■ STUDY PROTOCOLS

Administration Protocol of Radiotracer and P-gp Inhibitor. In the discussion around P-gp and other transporters, the main issue has often been whether a drug is altering P-gp function or if its own distribution into the brain is affected by P-gp. The first question has been answered by PET by performing scans with a known P-gp substrate tracer, for example, (*R*)-[^{11}C]verapamil, before and after intervention with the studied drug. To answer the second question, the studied drug has been labeled and PET scans have then been performed before and after intervention with a known P-gp inhibitor. The pharmacokinetic parameter of interest in most PET studies is the brain-to-plasma concentration ratio V_T, and this macro parameter is usually estimated by application of pharmacokinetic modeling of data obtained in PET scans where the tracer is administered as a single bolus.[19,23] These experiments have often resembled receptor studies in the respect that the second PET scan normally has been obtained at least 30 min after the P-gp modulating intervention was made. The effect of P-gp inhibitors has in several studies been shown to be very fast and thus leading to a rapid increase in radiotracer concentrations in the brain.[40,41,44,72] Also, P-gp function is rapidly restored when the inhibitor is eliminated. One study found that P-gp inhibition was more pronounced when Cyclosporine A was coinjected with the tracer compared to when Cyclosporine A was administered 30−37 min prior to tracer injection.[71] Thus, PET scanning at a relatively late time point after P-gp inhibitor administration may underestimate the effect of the inhibitor. It is possible to incorporate the dynamics of the P-gp inhibition and to PET scan at maximal inhibition when utilizing an infusion protocol of the PET tracer to obtain steady state concentrations in brain and plasma before administering the P-gp inhibitor or intervention drug.[40,41] A further advantage with using infusion designs and administration of a P-gp modulator during the scan is that the effect on P-gp function of also very small doses of P-gp inhibitors can be investigated without risking that the inhibitor has been completely cleared already at the start of the PET scan. V_T can then be estimated directly from the steady state levels before and after the intervention and the difference can be directly compared. The "two bolus" design and the "single infusion design" are shown in Figure 6.

Compared to the "two bolus" design, there are additional considerations to be made when planning infusion protocols with PET. Infusions are technically more difficult than bolus injections and require an infusion pump. The syringe containing the tracer has to be shielded to protect both personnel and PET scanner from radiation. In addition, it may not be possible to get the system into equilibrium within the time window of the scan. Thus, the pharmacokinetics (transport rate across the BBB) has to be known prior to designing an infusion protocol.

Intervention with P-gp Inhibitors in PET Studies. Most PET studies of P-gp have investigated alteration in function either due to a disease state or due to chemical inhibition. Alterations of P-gp function in disease and aging will be discussed in the next section. A number of different P-gp inhibitors have been developed during the last three decades.[87] The two most commonly used P-gp inhibitors in PET studies

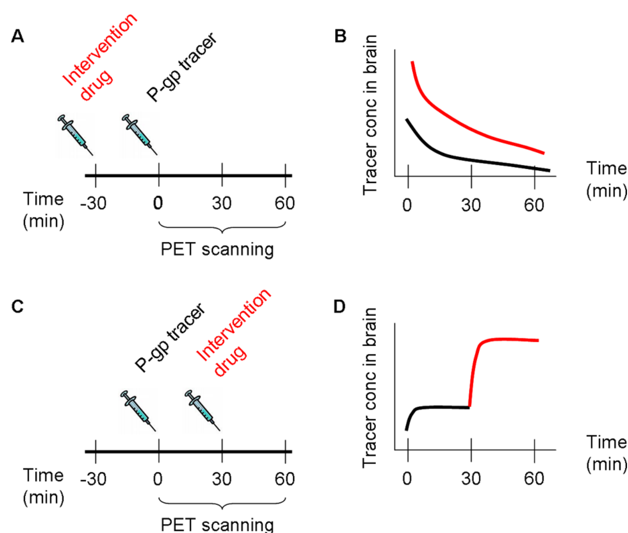

Figure 6. Different study protocols for studies of P-gp function with PET and illustrations of the resulting concentration profiles in the brain. In most published studies, the intervention drug, for example, a P-gp inhibitor, has been given prior to a bolus injection of a P-gp substrate tracer (A). Using this design, the effect of the P-gp modulation on brain concentrations of the tracer can be deduced by comparing treated (red line) and nontreated (black line) groups (B). The P-gp substrate tracer can also be given as an infusion prior to intervention with a P-gp modulator (C). In this case, the effect of the P-gp modulation can be estimated by comparing brain concentrations of the P-gp substrate tracer before (black line) and after (red line) the intervention (D). In this design, individuals act as their own control within a single scan.

for intervention are Cyclosporine A[9,29,40,41,50,62,88,89] and tariquidar.[42−44,55,72,90] Cyclosporine A is a so-called first-generation inhibitor which is also a substrate for P-gp transport. Cyclosporine A is a immunosuppressant drug, and although used in one clinical PET study[89] it cannot be recommended as a P-gp modulator in humans at higher concentrations. In fact, studies investigating the potential benefits of coadministration of Cyclosporine A and chemotherapy agents otherwise restricted from reaching their targets by P-gp have showed that severe toxicity is observed at doses lower than those required to inhibit P-gp in vivo.[91] Since 2007−2008, most PET studies of P-gp function have utilized the third generation inhibitor tariquidar. Even if tariquidar is associated with less risk compared to Cyclosporine A, tariquidar has not be administered in high doses to obtain complete P-gp inhibition in humans. Doses that have been administered i.v. range between 2 and 8 mg/kg, and these dose levels give submaximal inhibition of P-gp, although the highest dose of 8 mg/kg appear to result in almost complete inhibition.[28,55,72,90] Other inhibitors that have been used include the second generation (no immunosuppressant effect) P-gp inhibitor valspodar (PSC833)[77] and third generation inhibitors zosuquidar (LY335979),[92] elacridar (GF120988,)[44,76] and DCPQ which is structurally related to zosuquidar.[52,93]

■ P-GP FUNCTION IN DISEASE AND AGING

P-gp function has been studied in a number of CNS diseases: epilepsy, schizophrenia, Alzheimer's and Parkinson's disease, as well as in aging (Table 2).

Alzheimer's Disease. Central to Alzheimer's disease pathogenesis is beta-amyloid (Aβ), a 40−42 amino acid long hydrophobic and self-aggregating peptide. Aβ monomers gradually aggregate into soluble oligomeric assemblies and eventually into insoluble fibrils. Aβ fibrils are the main constituents of senile plaques which are a hallmark of Alzheimer's disease. The low-density lipoprotein receptor-related protein-1 (LRP1) is one of the transporters involved in the clearance of Aβ out of the brain at the abluminal side of the BBB.[94] However, to transport Aβ efficiently from the brain to the blood, a transporter on the luminal side of the BBB is also required. Recently, it was suggested that P-gp, indeed expressed at the luminal membrane, transports Aβ. The evidence comes from several different studies. In vitro assays have showed that P-gp transports Aβ and that inhibition of P-gp decreases this transport.[95] In mice, it has been shown that there is a correlation between P-gp expression and brain Aβ levels.[96,97] Clinical evidence includes a study which discovered that deposition of Aβ was inversely correlated with P-glycoprotein expression in the brains of elderly nondemented humans.[98] A PET study in Alzheimer's disease patients and age matched controls using (*R*)-[^{11}C]verapamil found that the Alzheimer's disease group had a 23% global increase in brain distribution of (*R*)-[^{11}C]verapamil compared to controls.[4] This indicated that P-gp function was compromised in the patient group. Some regional differences were also detected; for example, the increase was 33% in posterior cingulate and only 6% in cerebellum.

Table 2. P-gp Function in Disease and Aging[a]

condition	P-gp function	reference to clinical PET studies
Alzheimer's disease	↓	4
Parkinson's disease	↓ (only in advanced stages of disease)	3
epilepsy	↑ (only a trend, but supported by preclinical studies)	2
schizophrenia	↑	1
aging	↓ (only in males)	104−107

[a]All studies have used P-gp substrate tracer (*R*)-[^{11}C]verapamil.

Parkinson's Disease. Similar to Alzheimer's disease, Parkinson's disease is a neurodegenerative disorder characterized by death of dopamine generating cells in the substantia nigra. The reason for this cell death is largely unknown, but leads to uncontrolled movements and later to dementia. Bartels et al. have reported an increased (*R*)-[^{11}C]verapamil uptake in the frontal lobe in advanced Parkinson's disease patients compared to healthy controls suggesting a decreased P-gp function.[3] However, the same group of researchers also reported that there were no differences between controls and patients in the early stages of Parkinson's disease.[99] Thus, it is unlikely that P-gp function is contributing to Parkinson's disease, but rather that the observed decrease in the advanced stages of the disease is a consequence of the disease itself or by the long-term treatments that the patients are exposed to.

Epilepsy. The antiepileptic drugs that are available on the market today fail to properly control seizures in 30−40% of the epilepsy population. The "transporter hypothesis" postulates that pharmacoresistance may be caused by limited drug distribution across the BBB due to increased activity of multidrug efflux transporters, such as P-gp.[100] (*R*)-[^{11}C]-verapamil studies in rodent models of epilepsy have showed that the pharmacokinetics of (*R*)-[^{11}C]verapamil across the BBB is in general slower in epileptic rats than in controls.[42,51]

In line with the transporter hypothesis, Bankstahl et al. reported that (*R*)-[^{11}C]verapamil brain-to-plasma concentration ratios were lower in cerebellum and thalamus in 48 h post status epilepticus rats compared to controls but that differences were not significant in other brain regions.[39] The first clinical (*R*)-[^{11}C]verapamil study failed to detect statistically significant differences in (*R*)-[^{11}C]verapamil model parameters between epileptogenic and nonepileptogenic brain regions although there was a trend that epileptogenic regions displayed a lower (*R*)-[^{11}C]verapamil concentration than nonepileptogenic regions.[2] As discussed above, the brain concentrations of (*R*)-[^{11}C]verapamil are in general very low and may make it difficult to detect an upregulation in P-gp function as hypothesized in epilepsy and this might be a reason for the rather inconclusive results obtained in epilepsy models and patients. The study by Bankstahl et al.[39] did show that the regional differences and the differences between controls and epileptic rats became somewhat more pronounced when P-gp was inhibited by a low dose of tariquidar. [^{11}C]Flumazenil, a $GABA_A$ receptor antagonist and a moderate P-gp substrate,[66] has been used to study differences between epileptic and control rats with respect to both BBB pharmacokinetics and receptor binding.[101] This study did not indicate changes at the level of BBB but rather indicated that the differences observed between the two groups were caused by changes at the $GABA_A$ receptor level. To further investigate the role of P-gp in epilepsy antiepileptic drugs, mephobarbital and phenytoin have been labeled with ^{11}C.[69,102] [^{11}C]Phenytoin was found to be a moderate P-gp substrate as the brain-to-plasma concentration ratio increased by 45% after complete P-gp inhibition with tariquidar, but [^{11}C]mephobarbital brain concentrations were not influenced by P-gp function. [^{11}C]Phenytoin studies in epileptic rats are at the moment ongoing.

Schizophrenia. The pathological mechanism leading to schizophrenia is unidentified, but impaired integrity of the BBB has been implicated by some authors.[103] One study with (*R*)-[^{11}C]verapamil in patients with schizophrenia has been published so far and somewhat surprisingly reported an increased P-gp function in certain brain regions such as the temporal cortex, the amygdala, and the basal ganglia, that is, regions involved in schizophrenia.[1] The authors suggested that this upregulation may be due to long-term treatment with antipsychotic drugs, inflammation, or as a compensatory mechanism of BBB breakdown. Further, an increased P-gp function in schizophrenia may lead to pharmacoresistance in line with what has been suggested as a reason for refractory epilepsy.

Age and Gender. A decline in P-gp function with age could lead to accumulation of toxic substances in the brain and might be a mechanism by which age acts as the main risk factor for the development of neurodegenerative disease. The first (*R*)-[^{11}C]verapamil PET study investigating the effect of age on P-gp function showed a decline, measured as a increase in V_T by 15%. The study was performed in five elderly healthy volunteers (59−68 years) which were compared with a group of five younger healthy volunteers (21−27 years).[104] These findings were later underlined by Bauer et al. who showed that V_T was increased by 30% in elderly (69 ± 9 years) compared to younger healthy volunteers (27 ± 4 years)[105] and by Bartels et al. who showed that V_T was increased by 38% in elderly (60 ± 11 years) compared to younger healthy volunteers (24 ± 2 years). A recent (*R*)-[^{11}C]verapamil PET study investigated potential differences in P-gp function between young (24 ± 2 years), middle aged (46 ± 3 years) and old (63 ± 4 years) men and women.[106] The main findings were that P-gp function, measured as increased (*R*)-[^{11}C]verapamil brain-to-plasma concentration ratio, was decreased with age but only in men. The brain concentrations of (*R*)-[^{11}C]verapamil and thus P-gp function were similar in all three age groups in women. Further, it appeared that P-gp function was less efficient in young females compared to young males, but that these gender differences disappeared in the middle age and old age groups. The previous studies[104,105,107] had mainly included male healthy volunteers, and hence Assema et al., showed the importance of balanced male/female ratios in studies of P-gp function. In addition to these clinical studies, one (*R*)-[^{11}C]verapamil study in rhesus monkeys of different ages showed that P-gp function was decreased in very young individuals compared to adults.[64] Taken together, these clinical and preclinical studies indicate that P-gp function displays a bell-shaped curve over a life span with less efficient function in very young and elderly subjects.

■ CONCLUSIONS

PET imaging of P-gp function was performed for the first time in the late 1990s, and since then the number of available PET tracers has evolved continuously, and the use of PET in P-gp research has increased and diversified especially during the last 5 years. (*R*)-[^{11}C]Verapamil and [^{11}C]dLop have been the most frequently used P-gp substrate tracers for PET studies of P-gp function at the BBB. P-gp function studied clinically with (*R*)-[^{11}C]verapamil was found to be decreased in Alzheimer's disease, Parkinson's disease, and in the elderly. Although better suited to visualize decreased P-gp function, (*R*)-[^{11}C]verapamil studies have also indicated elevated P-gp function in epilepsy and schizophrenia. A number of labeled P-gp inhibitors have been investigated, but all of them act as substrates when used at tracer concentrations and hence are not useful for imaging P-gp expression at the BBB. However, they may have applications in other areas of PET imaging, for example, to distinguish tumors that overexpress P-gp from tumors that do not overexpress P-gp. In addition, it should be investigated if the "inhibitor" tracers may be preferable as substrate tracers over (*R*)-[^{11}C]verapamil and [^{11}C]dLop.

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Author Contributions

Stina Syvänen and Jonas Eriksson, surveyed the literature, wrote and prepared the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

5-HT, serotonin; [^{11}C]dLop, [^{11}C]desmethyl-loperamide; Aβ, amyloid-beta; BBB, blood-brain barrier; BCRP, breast cancer resistant protein; C, carbon; CNS, central nervous system; GABA, γ-aminobutyric acid; LRP1, low-density lipoprotein receptor-related protein-1; PET, positron emission tomography; P-gp, P-glycoprotein; SUV, standardized uptake value; V_T, volume of distribution

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on December 4, 2012, with minor errors in column 2 of Table 1. The corrected version was reposted on December 14, 2012.