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Monitoring the Function of the P-glycoprotein Transporter at the Blood Brain Barrier

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Monitoring the Function of the P-glycoprotein Transporter at the Blood Brain Barrier

Lara García Varela

The research reported in this thesis was carried out at the Department of Nuclear Medicine and Molecular Imaging of the University Medical Center of Groningen, Groningen, the Netherlands, at the Department of Nuclear Medicine and Molecular Imaging Group and at the Clinical Neurosciences Research Laboratory of IDIS Health Research Institute, Santiago de Compostela, Spain.

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PhD thesis

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the decision by the College of Deans.

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Chapter 1

General Introduction

Introduction

The Blood-Brain Barrier

The Central Nervous System (CNS) regulates the function of the whole human body and therefore, it needs to be well-protected. The CNS is covered by a particular microvasculature that separates the neural tissue from the blood and is termed the blood-brain barrier (BBB). The BBB provides nutrients to the brain and excretes harmful substances resulting from brain metabolism, moreover, it limits the entrance of a wide variety of compounds to the CNS (1,2). Thus, the main function of the BBB is to maintain the homeostasis of the CNS to ensure an adequate neural function (2,3).

These restricted properties can be achieved because the brain capillaries are continuous vessels formed of endothelial cells connected by tight junctions. These cells are surrounded by pericytes which are embedded in the basement membrane. All these elements are covered by the astrocytes' end-feet and form the neurovascular unit (4). Consequently, the brain endothelial cells present different characteristics than the endothelial cells located in the periphery. First, as previously mentioned, the endothelial cells are connected by tight junctions that restrict the paracellular transport across the cells. The vesicle-mediated transcellular transport (transcytosis) of molecules is also limited in the CNS endothelial cells compared with peripheral endothelial cells. Moreover, brain endothelial cells express many transporters: efflux transporters and specific nutrient transporters or solute carrier transporters (SLCs). The efflux transporters are mainly expressed at the luminal side and transport lipophilic compounds from the brain to the blood. On the other hand, the specific nutrient transporters move nutrients from the blood to the brain and also remove specific brain residues out of the brain (5). Most of these transporters need adenosine triphosphate (ATP) as energy fuel to move the compounds, thus a higher number of mitochondria are present in endothelial brain cells than in the periphery. In addition, lower expression of leukocyte adhesion molecules is found in brain endothelial cells which limit the entrance of immune cells to the CNS. All these characteristics restrict the entrance of substances in the brain and tightly regulate the homeostasis of the CNS (5).

Therefore, the BBB is also an obstacle for the delivery of CNS drugs, which impede the treatment of several brain disorders such as infectious diseases, psychiatric diseases, brain tumors, and neurodegenerative diseases (4,6). For this reason, one of the biggest challenges is to design drugs able to penetrate the BBB and achieve an adequate concentration inside the brain (7). Nowadays, many strategies have been proposed for the delivery of drugs to the CNS such as the use of

nanoparticles, microbubble-mediated focused ultrasounds, improve the lipophilicity of drugs, develop analogs of endogenous transporter ligand, and also the inhibition of efflux transport, which may pump out of the brain the treatments, to increase the drug concentration in brain tissue (7,8).

On the other hand, dysfunctions on the BBB properties are related to neurological diseases. Genetic mutations that affect the physiology of the BBB can induce neurological deficits (9). For instance, alteration in the SLC2A1 gene, which encodes the glucose transporter (GLUT1) located at the BBB causes decreases in glucose uptake and consequently microcephaly and developmental delay (De Vivo disease). Also, mutations in the SLS16A2 gene encoding the transporter for T3 thyroid hormone cause Allan-Herndon-Dudley syndrome. This syndrome is characterized by impaired neuronal development and functional deficits. Also, some brain disorders that are not associated with genetic mutations show BBB alterations. Alterations in the endothelial brain cells may precede and contribute to the development of Alzheimer's disease (AD). BBB breakdown in the hippocampus and a decreased function of efflux transporters located at the BBB was found in early AD patients (9). Moreover, patients with intractable epilepsy have shown an increase in the function of the P-glycoprotein (P-gp) transporter, the most studied efflux transporter at the BBB. These facts place the BBB as a critical player in various brain diseases (7,9).

The Efflux Transporters

Although many factors may contribute to the onset and progression of brain disorders, the efflux transporters or ATP-Binding Cassette (ABC) transporters' family play an important role. The ABC transporters represent one of the largest protein family and these transporters are mainly located in tissue barrier as the BBB and the blood-placenta barrier as well as in tissues involved in absorption and excretion functions such as the liver and kidneys (10,11). These transporters use ATP to drive substances from the brain back to the blood. Thus, their main function is to protect the brain from harmful exogenous substances and expel toxic endogenous compounds out of the CNS (12). Several compounds can be recognized by these transporters, although common substrates are usually lipophilic (13,14). Therefore, several small lipophilic compounds that should reach the brain by passive diffusion cannot cross the BBB because of the action of the efflux transporters. Thus, these transporters represent a great obstacle for the delivery of drugs into the brain and are associated with pharmaco-resistance to CNS treatments (1,15). On the other hand, alterations of ABC transporter function or expression have been related to various neurological disorders such as epilepsy, Alzheimer's disease, Parkinson's disease, multiple sclerosis among others (1).

Figure 1: P-gp transporter located at the BBB.

The P-glycoprotein (P-gp) or ABCB1, encoding by the MDR1 gene, is one of the most important and the best-known efflux transporter due to its clinical relevance (2,16) (Figure 1). Many factors such as diet, stress, and medication can alter the function or expression of this transporter (17). Increases in the P-gp function are associated with decreased drug efficacy (pharmaco-resistance). There are many drugs such as chemotherapeutics, antidepressants, antiepileptics, etc. that have a high affinity to the P-gp transporter (18,19). Therefore, these drugs are pumped out of the brain, reducing their drug efficacy. For instance, patients with intractable epilepsy have shown increased P-gp function and expression in the epileptogenic lesion, which restricts the entrance of antiepileptic drugs, and consequently, these patients are unsensible to the treatments (20). On the other hand, decreases in the P-gp function are related to the onset and progression of several neurodegenerative diseases. β -amyloid is also a substrate of the P-gp transporters and its accumulation inside the brain, which is the main hallmark of Alzheimer's disease, has been related to a decreased P-gp function (21,22). Other neurodegenerative disorders such as Parkinson's Disease or multiple sclerosis have also been related to an altered P-gp function or expression (1). Since the P-gp transporter plays an important role in different pathologies, it is of interest to measure its function and expression *in vivo*.

Positron Emission Tomography

Positron Emission Tomography (PET) is a molecular imaging technique that allows the quantification of biological processes *in vivo*. To this aim, a very low amount (trace amount) of a labeled compound (called radiotracer) is injected intravenously into the subject under study. This radiotracer is formed of a radionuclide and a molecule that shows a high level of specificity and selectivity towards a target site. For PET radiotracers the molecule must be labeled with a positron emitted radionuclide (e.g., fluorine-18 (^{18}F), carbon-11 (^{11}C), oxygen-15 (^{15}O), gallium-68 (^{68}Ga) or zirconium-89 (^{89}Zr)). The emission of a positron (β^+ decay) occurs with radionuclides

that are rich in protons when a proton turns into a neutron, a positron, and a neutrino. The positron is the antiparticle of an electron with the same mass but with a positive electric charge. When a radionuclide emits a positron, its magic number remains the same and the atomic number decreases in one unit. The emitted positron travels a short distance usually not more than 1-2 mm in tissue. However, this distance depends on the kinetic energy of an emitted positron and the density of the medium where it is emitted. During the travel, the positron loses its energy and it ends hitting an electron in a process called annihilation which results in two gamma rays (photons) of equal energy (511 keV) emitted in opposite directions (approximately 180° apart) that are detected by the PET detectors (23,24).

PET scanners are formed of multiple rings of gamma rays' detectors. When gamma rays hit the detectors, they activate the atoms of the scintillation crystals and produce visible light, and this light is converted into an electronic signal. This signal is amplified by photosensors (photomultiplier tubes or silicon photomultipliers in the next-generation PET scanners) that ensure that the amount of light detected is proportional to the energy of the absorbed photons. The PET system only registers the event when two photons are detected within a narrow time-window (coincidence detection). The line between these two detections is called the line of response (LOR) and allows to localize the emission of the positron within the field of view (FOV) (23,24).

Although thousands of coincidence events are detected during the PET scan, some erroneous detections are also registered and some events are also missed. Therefore, it is important to adequately calibrate the PET scanners and perform physical corrections after the collection of the PET data. The most important physical corrections are dead-time, attenuation, randoms, and scatter effect. After applying the physical correction to the data, a 3-dimensional image is created from the projected data by using different types of reconstruction algorithms. The most common approaches are the filtered back-projection and the iterative reconstructions. The acquired image represents the distribution of the radionuclide throughout the body of a subject and this image allows the generation of time-activity-curves (TAC) of specific regions or volume of interest (VOIs) (23,24).

Overall, PET experiments require the production of a radionuclide, the synthesis of a radiotracer, the intravenous injection of the radiotracer to a subject, the acquisition of a PET image that can be static or dynamic, the collection of blood samples to measure the radioactivity in the blood during the PET scan, corrections of the PET acquisition and reconstruction of the data, analysis of the PET data and measurements of radioactivity in the blood samples.

Production of PET radionuclides

Radionuclides used in PET are produced by accelerators or nuclear generators. Cyclotrons are the most common accelerators and they accelerate charged particles (usually H^+ or H^-) to a high velocity and in a circular trajectory. Cyclotrons can accelerate the particles alternating high-frequency voltages between the electrodes called “Dees”. The Dees present a magnetic field that is perpendicular to the electrode plane and between the Dees there is a gap with a uniform electric field. The particle is charged in the electric field and transport into one of the Dees. The particle then follows a half-circle trajectory due to the magnetic field that brings it back to the gap. The polarity of the electric field reverses each time the particle pass from one dee to another, thus every time the particle crosses the gap it gains energy. At the end, when the particle is highly accelerated, it is extracted from the circular path and hits the target. If the accelerated particle is a negative ion it is extracted with a carbon foil to remove the electrons, the resulting ion is positively charged, and it changes its trajectory in the magnetic field towards the target. On the other hand, positive ions are extracted from the circular path by a deflector that perturbs the magnetic field and directs the ion into the target. The radionuclides are formed by the reaction between the accelerated particle and the nuclei of the target molecule. For instance, fluoride-18 is produced by the nuclear reaction $^{18}O(p,n)^{18}F$, which indicates that the enriched water $[^{18}O]H_2O$ is bombarded by high energy protons (p) leading to the emission of a neutron (n) and an ^{18}F nucleus.

Another way to produce radionuclides is by the use of radionuclide generators. This mechanism uses a longer-lived parent radionuclide that decays to a daughter nuclide that can be separated from the parent easily. Generators facilitate the supply of short half-live radionuclides to hospitals and research facilities that do not have an on-site cyclotron facility (23,24).

Radiochemistry

Radiochemistry involves the chemical reactions to attach the radionuclide to the specific molecule to form the radiotracer. These radiotracers may be used to measure physiological parameters such as a specific transporter function, receptor density, blood flow, or rate of metabolism. The chemical reactions must be fast due to the decay of the radionuclide, especially when short half-life radionuclides are used. The radiosynthesis must be also robust and reproducible for easy production of the radiotracer. The reactions can be performed manually. However, since the radionuclide decays during the synthesis procedure, it is usually required to start with a high amount of radioactivity, thus the synthesis may be performed in closed lead-protected “hot cells” using an automatic synthesis module to reduce the radiation exposure (Figure 2). Another

important fact is the efficiency of the radiochemistry reaction, e.g., the amount of labeled compound at the end of the synthesis. Due to the decay of the radionuclide, these reactions are usually an inefficient process, particularly when using short half-life radionuclides. The molar activity (A_m) must be calculated before the use of the radiotracer and it is defined as the radioactivity per total amount in a mole of a compound and it is expressed in Bq/mol or GBq/ μ mol (25). Thus, it measures the ratio between the amount of labeled compound and the total amount of the unlabeled compound. Low A_m can be found when during the production process the non-radiolabeled material is not adequately removed from the labeled product. Then, the high amount of unlabeled compound may interfere in the system or cause a pharmacological effect that may alter the kinetics of the labeled compound (radiotracer) or it can also saturate the target under evaluation and then cause a reduction in the PET signal. Therefore, it is important to measure the A_m of the radiotracer before the injection in the living subject (23,24).

Figure 2: Hot cell (left) and robot for radiosynthesis (right) located in the R&D lab in the Nuclear Medicine and Molecular Imaging department at the University Medical Center of Groningen.

Radiochemistry with fluorine-18

The fluorine-18 can be obtained as: [^{18}F]fluoride ($[^{18}\text{F}]^-$) or fluorine-18 gas ($[^{18}\text{F}]\text{F}_2$). The fluoride-18 form allows the introduction of the ^{18}F -atom by nucleophilic substitution reactions and the fluorine-18 gas by electrophilic substitution reactions of electron-rich systems. The production of fluoride-18 by the nuclear reaction $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ using ^{18}O -enriched water as the target, is relatively easy, and [^{18}F]fluoride can be produced in a large amount. However, the production of [^{18}F]F $_2$ is more complicated, although can be achieved by two different nuclear reactions the $^{20}\text{Ne}(\text{d},\alpha)^{18}\text{F}$ or $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ using ^{20}Ne or enriched [^{18}O]O $_2$ as target gases, respectively (24).

Nucleophilic fluorinations with [^{18}F]fluoride are predominantly used in PET-tracer synthesis. First, the cyclotron-produced [^{18}F]fluoride in an aqueous solution is passed through an anion exchange cartridge to trap the [^{18}F]fluoride. The elution of the [^{18}F]fluoride from the anion exchange is done with a phase transfer catalyst (Kryptofix 222) and a weak base such as K_2CO_3 , thus, the nucleophilicity of the ion [^{18}F]fluoride is enhanced. In an aqueous solution, the [^{18}F]fluoride is strongly hydrated due to its electronegativity and it can form hydrogen fluoride. Therefore, the water has to be removed to facilitate the nucleophilic substitution reactions. Thus, after the elution, the [^{18}F]fluoride is dried via repeatedly azeotropic distillation with acetonitrile. This results in a naked highly reactive [^{18}F]fluoride due to the weak base used in the elution. Also, the solubility of the [^{18}F]fluoride in a polar aprotic solvent is improved due to the presence of the Kryptofix 222. Aliphatic ^{18}F -substitutions require the presence of a good leaving group such as Cl, Br, tosylate or mesylate groups. These reactions are usually performed at high temperatures and in polar aprotic solvents (ACN, DMF, DMSO). With regards to nucleophilic aromatic substitution, a good leaving group is also required but also the presence of an activated electron-withdrawing group in ortho- or para- position relative to the leaving group. This reaction usually requires high temperatures and higher-boiling solvents as DMSO, DMF, and DMA. Next, protecting groups used to eliminate acid protons (such as the ones in hydroxyl, thiol, carboxyl, and amines groups) have to be removed from the molecule. After the reaction, the final product has to be purified by solid-phase extraction or preparative HPLC (24).

PET acquisition

PET acquisition can be static or dynamic. Static PET images consist of a single PET image that represents the average detected radioactivity over a certain period of time. In general, static images are performed some period after the injection of the radiotracers (around 30 minutes after). It is expected that during the static PET scan the tracer has reached the equilibrium and the radioactivity detected represents the process of interest. However, static PET images do not allow a quantitative interpretation of the data. The most common parameter extracted from the static PET images is the Standardized Uptake Value (SUV). The SUV is a semi-quantitative parameter and it is defined as the radioactivity concentration in the tissue corrected by the injected dose and the bodyweight of the subject and it is calculated as described in the following equation:

$$SUV = \frac{\text{Radioactivity concentration in tissue (KBq/ml)}}{\text{Injected dose (MBq) / Body weight (Kg)}}$$

Figure 3: SUV values are composed of different radioactive signals.

The distribution of the tracer in vivo is a dynamic process and the static images only capture a specific moment of that process. The radioactivity detected at that moment and calculated as SUV is composed of different signals that may represent the specific binding of the tracer and the non-specific binding. Static images assume that the distribution of the tracer at late time-points is mostly related to the specific binding of the tracer. However, this may not be true for all the PET tracers and acquisition protocols. Moreover, SUV measurements can be affected by changes in the amount of tracer available in the blood due to changes in the metabolism of the tracer or binding to plasma proteins. Also, changes in the blood flow or in the unspecific tissue binding can affect the SUV measurements. Thereby, although SUV measurements are commonly used in clinical settings due to their simplicity, they may not represent adequately the underlying biological process (23,24).

Dynamic PET acquisition records the distribution of the tracer over a certain time, immediately after the injection of the tracer. From these images, an estimated concentration of radioactivity can be derived from different regions of interest as a function of time called tissue time-activity curves (TACs). As mentioned before, several factors contribute to the radioactive signal detected by the PET scanners: the radioactivity measured in the regions of interest which include the specific binding of the tracer to the target and other non-specific binding and it also includes the radioactivity in the blood compartment (blood volume). To extract more information from this data, mathematical models can be applied to know the real amount of tracer binding to our target or representing the physiological process of interest. To this aim, the radioactivity concentration derived from the PET images has to be related to the radioactivity in the input function. The input function represents the available radioactivity to be delivered into the region of interest. To obtain this input function, blood samples must be collected during the PET scan at different time points after the tracer injection (Figure 4). Since the radiotracer can be attached to the red cells in the blood, and therefore, this fraction of tracer is not available to enter the region of interest, the plasma needs to be separated by centrifugation of the samples. Moreover, it is also important to quantify the radioactive metabolites in plasma, since the radiotracer can be metabolized after its

administration to a subject. Furthermore, the binding of the tracer to plasma proteins can also be analyzed. After all these corrections, the metabolite-corrected radioactivity concentration in plasma is obtained and subsequently used as input function. Recently, image-derived input functions obtained from large blood pools such as the heart or aortic segments have also been proposed as an alternative to blood sampling (23,24,26).

Figure 4: Dynamic-PET scan for small animals with blood sampling.

Next, physical corrections such as dead-time, attenuation, scatter, and randoms are applied to the PET images to avoid erroneous or missed events, as previously mentioned. Afterward, PET images are reconstructed and co-registered to anatomical images (Magnetic Resonance Imaging (MRI) or Computed Tomography (CT)), which allow the localization of specific regions. Finally, a mathematical model is applied to the radioactivity concentration in the tissue measured by the PET cameras and the metabolite-corrected plasma radioactivity (input function) to describe the movement of the tracer between the plasma and different compartments or volumes of interest. However, TACs from a tissue without the interested target (reference tissue) can also be used as input function. The mathematical models that use this type of input function are called reference tissue models (23,24,26).

Quantification methods: kinetic modeling.

Compartmental Models

The kinetics of the tracer are usually described with compartmental models. Compartmental models assume that the tracer is homogeneously distributed between the compartments. Tracer exchanges between compartment can be described using differential equations, as follow:

$$\frac{dC_T(t)}{dt} = K_1 C_a(t) - k_2 C_T(t)$$

The changes in the tracer concentration in the tissue ($C_T(t)$) during a certain period of time equals the K_1 (Influx rate constant) multiplied by the concentration of the tracer in arterial plasma ($C_a(t)$) minus the k_2 (efflux constant) multiplied by the concentration of the tracer in tissue. After applying the Laplace transformation, the equation can be solved as follow:

$$C_T = K_1 C_a(t) * e^{(-k_2 t)}$$

The * symbol indicates a convolution of two functions which is a mathematical operation that results in a third function that expresses how the shape of the first function is modified by changes in the second one. Here, the tracer concentration in the plasma as a function of time convoluted with the response in the tissue as a function of time to give tracer concentration in the tissue as a function of time. PET scans provide the tracer concentration in the tissue (C_T) and the blood samples draw during the PET scan measures the tracer concentration in arterial plasma (C_a). After collecting various measurements of C_T and C_a , if the data can be fitted by the simplest compartmental model, the 1-Tissue Compartment Model (1-TCM), the rate constant K_1 and k_2 can be estimated by applying nonlinear least-square fitting to the previous equation.

The influx constant or K_1 represents the entrance of the tracer from the plasma to the tissue and it depends on the blood flow and the extraction fraction of the tracer. The extraction fraction depends on the permeability of the tracer across the membranes and the surface area of the tissue of interest. The efflux constant or k_2 measures the clearance of the tracer from a specific tissue. After a certain period of time, the concentrations of the tracer in the plasma and tissue are constant and the system is considered to be in equilibrium. Then, the ratio of these two concentrations (C_T/C_a) can be calculated, called the volume of distribution (V_T) ($V_T=K_1/k_2$).

However, the tracer can be in different states such as free in the tissue, bound to a non-specific receptor (protein or enzyme), or bound specifically to the target of interest. To distinguish among these different states, the compartmental model can be extended. Usually for modeling the reversible binding of a tracer, the 2-Tissue Compartmental Model (2-TCM) is used. In this case, the tracer is transported from the plasma to the first tissue compartment where the tracer is usually free or bound to a non-specific receptor (in a small fraction), and from the first tissue compartment, it moves to the second tissue compartment where the tracer is specifically bound to the target of interest (Figure 5). As in the 1-TCM, the K_1 and K_2 measure the entrances and clearance of the tracer from the plasma to the first compartment. The rate constants K_3 and k_4

describe the exchanges between the two tissue compartments. The most important estimated parameters in the 2-TCM are the V_T calculated as $V_T = \left(\frac{K_1}{k_2}\right) \left(1 + \frac{k_3}{k_4}\right)$ but also the specific binding to the receptor called the binding potential non-displaceable (BP_{ND}) which is calculated as $BP_{ND} = \frac{k_3}{k_4}$.

In the case of tracer with irreversible binding or that remains trapped in the tissue during the PET scan, irreversible models have to be applied. The most common one is the Irreversible 2-TCM. In this case, the k_3 represents the rate of trapping of the tracer or the rate of tracer internalization, and consequently, the k_4 is equal to zero. The irreversible 2-TCM allows the calculation of the net uptake rate or net influx rate (K_i) as $K_i = \frac{K_1 k_3}{k_2 + k_3}$.

From these models, we can differentiate between macroparameters as the V_T , BP , and the K_i and microparameters as the rate constants (K_1 , k_2 , k_3 , and k_4). Macroparameters are calculated by combining the rate constants and show better reproducibility (more robust than the separated rate constants) (23,24,27).

Figure 5: Representation of 1-Tissue Compartmental Model (A) and 2-Tissue Compartmental Model (B).

Linearization Methods and Graphical Analysis

The most common and simple methods to apply to the PET data are the linearization methods, which consist of a mathematical model that transform the measured data into a straight-line plot after a certain period of time (t) needed to reach the equilibrium between the radioactivity in the tissue and the radioactivity in the plasma. The slope and intercept generated by linear regression analysis have a physiological meaning.

Figure 6: Examples of a Logan Plot.

The most frequently used linearization methods are the Logan and Patlak graphical analysis (Figure 6). Logan analysis is used to fit the data of reversible tracer, in this case, the slope of the plot represents the volume of distribution (V_T) of the tracer which is the ratio of the concentration of radiotracer in an interested tissue to that in plasma. Patlak analysis is used to fit the data of irreversible tracers e.g., tracers that bound irreversibly to the receptor or are trapped in a compartment. In this case, the slope of the Patlak plot estimates the net uptake rate (K_i). Contrary to compartmental models, linearization requires less computational power and only estimates macroparameters. Although the estimated macroparameters are more robust than the parameters derived by the compartmental models, the graphical analyses do not consider the blood contribution in the tissue compartment and are more sensitive to noise data. Therefore, all these restrictions and, also, violations of the steady-state of the system can lead to erroneous estimations of the V_T and K_i .

Linearization methods are especially useful in voxel-by-voxel analysis and for the generation of parametric images. Parametric images estimate individually for each pixel the parameter of interest which allows the visual assessment of parameter differences across the brain and allows the assessment of the heterogeneity within tissues. Since linearization methods are simpler than the nonlinear least-square fitting used in compartmental models, macroparameters at a voxel level can be rapidly calculated. However, Logan and Patlak graphical analyses do not allow the estimation of microparametes (rate constants), thus, other approaches have been proposed as the use of basis function methods. These methods allow the calculation of rate constants and include the blood compartment in the analysis (23,24,27).

Reference Tissue Models

As previously described TACs from a tissue without the interested target (reference tissue) can also be used as input function. Reference tissue models are used as alternative kinetic models to avoid blood sampling during the PET scan. Therefore, measurements of the blood samples and metabolites analysis are not needed and consequently, the imaging protocol is simplified. However, these models are based on several assumptions and restrictions. For instance, the reference tissue used as input function has to be devoid of tracer binding sites, and thus the non-displaceable volume of distribution of the region of interest should be equal to that in the reference region, e.g., $K_1/k_2 = K_1'/k_2'$.

When it is not possible to differentiate between the free tracer, the non-specific, and the specific binding in both the target regions and the reference region, the reference tissue model can be reduced into the simplified reference tissue model (SRTM). Moreover, linearization methods can also be used with a reference region as input functions. The reference region logan plot calculates the V_T of reversible tracer and the K_i^{Ref} can also be estimated using the Patlak with reference region as input.

Reference tissue models should always be compared with those using plasma radioactivity as input function before the validation of the method (23,24,27).

Radiotracers for measuring the P-gp transporter function

In 1996, a racemic mixture of [^{11}C]verapamil was used for the first time to evaluate the P-gp function (28). Since that moment, the P-gp function has been extensively studied under different conditions and in different species (21,29–36). The racemic mixture complicates the pharmacokinetic quantification of the radiotracers, thus (*R*)-[^{11}C]verapamil was selected as the most adequate radiotracer due to its lower rate of metabolism and lower affinity to calcium channels. One of the main drawbacks of [^{11}C]verapamil is its extensive in vivo metabolism resulting in the formation of radioactive metabolites that are also P-gp substrates (37). The presence of radioactive metabolites can interfere with the PET signal and lead to quantification errors (38,39). Another P-gp PET tracer was synthesized with improved metabolic activity than the (*R*)-[^{11}C]verapamil, the [^{11}C]-N-desmethyl-loperamide ([^{11}C]dLop) (40).

Nowadays, the most commonly used PET tracers for imaging P-gp function at the BBB are (*R*)-[^{11}C]verapamil and [^{11}C]dLop. These tracers have been extensively used in small animals and humans (39). Since these tracers act as strong P-gp substrates, at baseline condition, when the P-

gp function is working adequately, the brain uptake of the tracers is very low. If the P-gp function decreases as it may happen in Alzheimer's disease patients or if the P-gp is inhibited, the brain uptake of these tracers increases. However, in those cases where the P-gp function is increased such as in patients with intractable epilepsy (20), the brain tracer uptake may decrease. Since the brain uptake of the tracer is already very low at baseline, these tracers are not able to measure increases in the P-gp function (41,42).

Current used P-gp PET-tracers measure the functionality of the P-gp transporters but cannot assess their expression levels. Thus, researchers are also focused on the development of P-gp tracers able to measure the P-gp expression. The first approach was the use of P-gp inhibitors, since inhibitors may attach to the ligand-binding site and block it (17). Therefore, P-gp inhibitors as tariquidar, elacridar, and laniquidar were radiolabelled with carbon-11 (43) (44) (45). Unfortunately, these P-gp inhibitors at low concentrations (tracer amount) were recognized as substrates by the P-gp transporters rather than inhibitors. As a result, the tracers were pumped out of the brain causing a low brain tracer uptake as occurs with the strong P-gp substrates PET tracers (42,46). Recently, a new molecule called [^{18}F]4FIMPTC developed by Verbeek et al has been identified as a promising PET tracer for measuring the P-gp expression. The studies performed in rats and mice suggest that this tracer binds to the P-gp transporter and may allow measurement of the P-gp expression in vivo but additional experiments are needed to confirm these results (47).

Many strategies have been explored to overcome a low baseline uptake of radioactivity in the brain. Lately, the approach was focused on the use of weak P-gp substrates which may show lower affinity to the transporter as tracers. This approach may lead to a higher baseline brain uptake which may facilitate the assessment of increases in the P-gp function. To date, several radiotracers such as [^{11}C]phenytoin, [^{11}C]metoclopramide, and [^{18}F]MC225 have already been identified as weak P-gp substrates and their kinetics are being evaluated (48–51). [^{18}F]MC225 has been synthesized by Savolainen et al. (52) and it is the radiotracer used in this thesis to monitor the P-gp function at the BBB of different animals species. This tracer has the potential to become the first radiofluorinated tracer able to measure increases and decreases of the P-gp function due to its adequate kinetic properties.

Outline of the thesis

The thesis aims to assess the properties of the novel P-gp PET tracer [^{18}F]MC225 under different conditions and in various animal species. To this aim, the kinetics of [^{18}F]MC225 were evaluated after the inhibition and the induction of the P-gp transporter function. The final aim is to provide sufficient information such as the ability of [^{18}F]MC225 to measure increases in the P-gp function

and to confirm its promising properties in a different species to support the translation of this novel PET tracer to the clinical settings.

First, **Chapter 2** introduces the recent advances regarding the PET imaging of ABC transporters at the BBB. The chapter describes the properties of different ABC transporters and their implication in several brain disorders such as neurodegenerative and psychiatric diseases as well as in drug resistance processes. Moreover, it provides an overview of currently available radiotracers for quantification of the function of different ABC transporters, in particular the P-gp transporter.

Since it is of interest to perform longitudinal PET studies with [^{18}F]MC225 to evaluate the P-gp function, the reproducibility of the [^{18}F]MC225 PET scans is evaluated in **Chapter 3**. Moreover, PET imaging of small animals involves the use of anesthesia to immobilize the subject during the scan, therefore the effect of isoflurane-anesthesia on the P-gp function is also assessed in this chapter.

To verify the promising kinetic characteristics of [^{18}F]MC225, its properties are evaluated in a species more closely related to humans than rodents before the transition of the tracer to the clinical settings. To this aim, **Chapter 4** describes the pharmacokinetic evaluation of [^{18}F]MC225 in three non-human primates before and after the inhibition of the P-gp function.

Furthermore, although the kinetics of the gold standard P-gp PET tracer, (*R*)-[^{11}C]verapamil, have been extensively studied in small rodents and humans, **Chapter 5** evaluates the pharmacokinetics (*R*)-[^{11}C]verapamil in non-human primates. The aim is to establish (*R*)-[^{11}C]verapamil as a reference P-gp tracer for comparison of newly developed P-gp PET tracers in non-human primates, a species closely related to humans to obtain a proper reference for the [^{18}F]MC225 studies.

Next, **Chapter 6** directly compared the properties of [^{18}F]MC225, with the properties of (*R*)-[^{11}C]verapamil in non-human primates. To this aim, the 30-min PET data of both tracers are fitted to a 1-TCM following the recommendations in previous publications. This chapter compares the kinetics values between tracers at baseline and after P-gp inhibition conditions. Furthermore, regional differences between tracers and simplified methods to measure the P-gp function are discussed.

Chapter 7 describes a new approach to produce [^{18}F]MC225 by one-step synthesis. The development of a new precursor that allows the production of [^{18}F]MC225 via direct radiofluorination is also described. This study aims to develop a faster and more robust radiosynthesis to facilitate its automatization and incorporation into the GMP facilities.

Finally, **Chapter 8** evaluates the effect of a novel P-gp inducer (MC111) on the P-gp transporter function. P-gp inducers act by increasing the P-gp function and thus, they may be used as alternative treatments of neurodegenerative diseases in which the P-gp function is decreased. The aim is to assess the ability of MC111 to increase the P-gp function in vivo at the BBB in healthy rats and to verify the ability of the radiotracer [^{18}F]MC225 to measure increased P-gp function.

Chapter 9 presents the concluding remarks and future perspectives of the thesis and **Chapter 10** provides a brief overview of the whole thesis, highlighting the most important outcomes.

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Chapter 2

PET Imaging of ABC Transporters at the Blood Brain Barrier

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Abstract

The function of ATP Binding Cassette (ABC) transporters at the blood-brain barrier (BBB) is to protect the brain from toxic compounds. Additionally, they play a crucial role in the onset and progression of several central nervous system (CNS) diseases as well as in drug resistance. Many compounds were identified as substrates, inhibitors, inducers or activators for ABC transporters, causing important drug-drug interactions. PET imaging represents an excellent tool for assessing the function and expression of ABC transporters. Over the last years, many PET tracers with different characteristics have been developed, mainly for measuring P-glycoprotein (P-gp) function at the BBB. Although (*R*)-[^{11}C]verapamil or [^{11}C]N-desmethyl-loperamide are considered as the “gold standard” P-gp tracers, they have several drawbacks such as its high affinity to P-gp which limits its use for assessing P-gp increased function. Therefore, PET tracers with lower affinity to the transporter have been developed and studied in different species. The assessment of ABC transporters by PET imaging can provide new insight into the physiology and pathophysiology of different CNS diseases and may open new avenues for therapies. Moreover, PET can be used for screening the affinity of new entities toward various ABC transporters and thus enhance the development of CNS drugs.

Keywords: ATP Binding Cassette transporters, PET tracers, P-glycoprotein, Positron Emission Tomography.

ABC Transporter Expression at the Blood-Brain Barrier in Neurological Diseases

Blood-brain barrier

The function of the human brain requires a strictly controlled environment. The blood-brain barrier (BBB) plays an important role in maintaining homeostasis of this micro-environment by protecting the brain from neurotoxic substances since it functions as a biochemical and physical barrier (1). This barrier was first described by Paul Ehrlich, who found that trypan blue injections in the peripheral blood circulation did not reach the spinal cord and brain in rabbits (2). Nowadays, we know the BBB consists of a monolayer of non-fenestrated brain microvessel endothelial cells connected by tight junctions, which limits transport by passive diffusion across the BBB. Only small lipophilic molecules and drugs with molecular weights less than 400 Da and less than eight hydrogen bonds can cross the barrier by passive diffusion (3).

For compounds that cannot enter the brain by passive diffusion due to their ionization, hydrophilicity or size, membrane transporters at the endothelial cells can facilitate the transport across the BBB (4). Apart from the limited transport by passive diffusion, the BBB also presents numerous efflux transporters that carry a wide variety of molecules from the brain to the blood. The best known efflux transporters are the ATP binding cassette transporters (ABC transporters). These transporters play an important role in protecting the central nervous system (CNS). In this chapter, we will focus on the ABC transporters, with a particular emphasis on P-glycoprotein and its involvement in several neurological disorders, such as Alzheimer's disease, Parkinson's disease, and epilepsy.

ABC transporters

The ABC transporter superfamily represents the largest family of transmembrane proteins and consists of about 50 members classified into seven different subfamilies. These transporters are ATP-dependent and use this energy to drive the transport of various endogenous and exogenous molecules across cell membranes (5,6). They play a significant role in various CNS disorders and their pharmaceutical treatments in two different ways: First, many pharmaceuticals used as therapeutic drugs in CNS diseases are substrates of ABC transporters, which limits their access to the brain. Second, the expression and function of ABC transporters may be altered in certain CNS diseases, either as a cause or a consequence of the disease, resulting in altered drug entry in the CNS (see table 1).

Table 1: CNS diseases involving impairments in ABC transporters.

CNS Disease	ABC transporter involved	The main change in ABC Transporters activity	Reference
Alzheimer's disease	ABCA1, ABCA2, ABCB1, ABCG2	Decreased P-gp, BCRP and MRP1 expression and function	(7–13)
Parkinson's Disease	ABCB1	Decreased P-gp expression and function	(14–17)
Epilepsy	ABCC1, ABCCs, ABCG2	Increased P-gp, BCRP, MRP1 and MRP2 expression and function	(18–26)
Brain tumors	ABCA1, ABCB1, ABCC1, ACC4, ABCG2	Increased P-gp, BCRP and MRP1 function and expression	(27–33)
Ischemic stroke	ABCB1, ABCG2, ABCC1, ABCC5	Increased P-gp, BCRP and MRP1 expression	(34–37)
Multiple sclerosis	ABCB1, ABCD3, ABCG2	Decreased P-gp expression	(38–40)
Amyotrophic lateral sclerosis	ABCB1, ABCG2	Increased P-gp and BCRP activity	(41–43)
HIV-encephalitis	ABCB1, ABCC1, BCC4, BCC5, ABCG2	Increased BCRP and MRP1 expression	(44,45)
Lysosomal storage diseases	ABCC4	Increased MRP4 function	(46)
Depression	ABCB1	Increased P-gp function	(47,48)
Schizophrenia	ABCB1	Decreased P-gp expression	(49)
Creutzfeldt-Jakob disease	ABCB1	Decreased P-gp expression	(50)

P-gp, also known as ABCB1, was the first ABC transporter described and has by far been the most studied. This transporter is found at the luminal side of the endothelial cells at the BBB and acts as an efflux transporter for various structurally different substrates (see table 2). Besides P-glycoprotein, other ABC transporters with a similar function exist. ABCG2 (or Breast Cancer Resistance Protein BCRP), ABCC1 (or Multidrug Resistance Protein MRP1), ABCC4, and ABCC5 are also localized at the luminal side of the human brain capillary endothelial cells of the BBB (see figure 1). ABCC6, ABCC11, and ABCC12 are also present in the microvasculature of the brain, albeit at lower expression levels (51).

Table 2: Examples of drugs that act as substrates of P-gp, BCRP and MRP1 transporters (updated from (52,53)).

ABC Transporter	Drug class	Substrates drugs
P-gp	Cytostatic	Vinblastine, vincristine, doxorubicin, paclitaxel, methotrexate
	Immunosuppressant	Cyclosporine A, tacrolimus
	Corticoids	Dexamethasone, aldosterone, cortisol
	Analgesics	Morphine, Methadone, Fentanyl
	Antiretroviral HIV	Ritonavir, indinavir
	Anthelmintic	Ivermectin
	Antidiarrheal	Loperamide
	Antiemetic	ondansetron
	Antibiotic	Erythromycin, tetracyclines, rifampicin
	Antiarrhythmic	Amiodarone
	Cardiac glycoside	Digoxin
	Calcium channel blocker	Verapamil, nifedipine, diltiazem
	Beta-blocker	Propanolol
	Statin	Lovastatin, simvastatin
	Antiepileptic	Phenytoin, carbamazepine, phenobarbital
	Antipsychotic	Chlorpromazine
	Antidepressant	Paroxetine, amitryptiline, venlafaxine
BCRP	Cytostatic	Anthracyclines, mitoxantrone, etoposide, Erlotinib, gefitinib
	Antiretroviral HIV	Zidovudine, lamivudine
	Antibiotics	Ciprofloxacin, Norfloxacin
	Immunosuppressant	Cyclosporine A, tacrolimus
MRP1	Cytostatic	Etoposide, vincristine, doxorubicin, methotrexate
	Immunosuppressant	Tacrolimus, cyclosporine A
	Antiretroviral HIV	Saquinavir, ritonavir
	Antiepileptic	Levetiracetam, phenobarbital
	Antibiotic	Azithromycin, ciprofloxacin

Figure 1: ABC transporters in the human endothelial cell at the BBB update from (52,54).

P-glycoprotein

P-glycoprotein (P-gp) or ABCB1, encoded by the MDR1 gene was the first ABC transporter discovered and remains the most studied one. This 170 kDa membrane protein is located between the cytoplasm and the periplasm and consists of a single chain with two homologous parts. At the intracellular side of the two homologous parts, six transmembrane (TM) helices and two ATP nucleotide binding domains (NBDs) are present. During the efflux of a substrate, translational and rotational motions in P-gp take place: ATP causes a change in the TM helix, which will catalyze the efflux of the P-gp substrate through the transmembrane domain (TMD) and lipid bilayer.

P-gp is involved in the efflux of a broad range of structurally different substrates including analgesics, antihistamines, and antibiotics and is therefore thought to play an important role in drug resistance (55) (see table 2). Besides the efflux of many pharmaceuticals, P-gp transports also endogenous compounds such as amyloid- β and, therefore, may play an important role in the pathophysiology of Alzheimer's disease.

BCRP

BCRP is encoded by the ABCG2 gene located on chromosome 4q22. This transporter was first discovered in 1998 by Doyle and colleagues in a multidrug-resistant human breast cancer cell line

(56). BCRP consist of a polytopic TM protein with 655 amino acids. It differs from P-gp in its structure since it is a half ABC transporter, consisting of only one nucleotide-binding domain and one membrane-spanning domain (MSD) (57). BCRP is responsible for the efflux of more than 200 different substrates, including chemotherapeutics, tyrosine kinase inhibitors, antivirals, carcinogens (see table 2) and also physiological substances, such as uric acid and estrone-3-sulfate. This makes BCRP one of the most important efflux transporters at the BBB. Although the substrate and inhibitor specificity of BCRP show some overlap with P-gp and MDR1, they are not completely identical, as can be explained by the different protein structures and working mechanisms (58).

MRP family and MRP1

Besides P-gp and BCRP, members of the Multidrug Resistance Protein (MRP) family are efflux transporters at the BBB. The transporters in this family are less studied and characterized than P-gp and BCRP and their function at the BBB is less clear, although it is known that the MRP family is responsible for the efflux of a diverse range of compounds including xenobiotics and endogenous substrates. Subfamily ABCC, which expresses the MRP transporters contains 12 proteins, and nine of these are known to be involved in ATP dependent transmembrane efflux of drugs in various cells and tissue types in the human body. Proteins in the MRP subfamily share common structural features including multiple TM helices in membrane-spanning domains (MSD) and NBDs at the intracellular side for the binding of ATP. MRP1, MRP2, MRP3, MRP6, and MRP7 have an additional NH₂-proximal MSD, and therefore they are called the “long MRPs” (59). The first of the nine drug-transporting MRPs is MRP1, which is encoded by the ABCC1 gene on chromosome 16p13.1 and is the most studied and best-known MRP family member. It was discovered by Cole et al. in 1992 as a cause of multidrug resistance in a drug-selected human lung cancer cell line (60) and it is because of this broad substrate specificity (table 2) that MRP1 continues to be of considerable interest in preclinical and clinical studies.

Alzheimer's disease

The incidence of neurodegenerative diseases has increased significantly over the past few decades because the life expectancy of the human population has increased. In 1906 Alzheimer's disease was first described by Alois Alzheimer as a particularly severe disease process of the cerebral cortex (61,62). Clinically Alzheimer's disease is characterized by a decline in memory, language, and cognitive skills, which affects the patient's ability to perform everyday activities (63). Alzheimer's disease is an irreversible and incurable condition. The underlying cause of these

symptoms can be found in the destruction of neurons in brain regions involved in the cognitive function. Although the exact pathogenesis of Alzheimer's remains unclear, hallmarks of AD are the accumulation of neurotoxic amyloid- β ($A\beta$) plaques in the extracellular compartment of the brain, inflammation, accumulation of hyper phosphorylated tau in intracellular neurofibrillary tangles and neuronal loss (64). The amyloid hypothesis suggests that this accumulation of $A\beta$ is caused by an imbalance of overproduction and impaired clearance, and this may be the key element triggering neuronal degeneration (65). Although no significant association was found between ABCB1 polymorphisms and the risk of developing AD in humans, P-gp is thought to play an important role in the pathophysiology of AD since this efflux transporter facilitates transport of $A\beta$ out of the brain (66,67). Various studies have reported an inverse correlation between $A\beta$ deposition and cerebrovascular ABCB1 expression (68,69).

Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by clinical symptoms of uncontrolled movement and in a later stage by dementia. The symptoms of this disease are caused by the death of dopamine generating cells in the substantia nigra. The reason for this cell death is unknown, although it is known that exposure to neurotoxic pesticides is a risk factor for the development of PD (70–72). Pesticides are a group of compounds with various mechanisms of action and pharmacokinetic properties, including fungicides, herbicides, and insecticides. In epidemiologic studies in humans, toxicity models in animals and in vitro studies, exposure to pesticides was shown to be associated with an increased risk of the development of PD. An alteration of the BBB transport of such neurotoxic pesticides has been proposed as an underlying pathophysiological mechanism that may lead to PD. Since P-gp has an extensive range of substrates, P-gp may be involved in the removal of neurotoxic pesticides out of the brain. However, it is still unknown if P-gp is truly involved in this transport, although multiple studies did investigate the association between ABCB1 and PD. In only a few studies an association between ABCB1 alterations and Parkinson's disease was found, especially in later stages of the disease (73–77).

Epilepsy and antiepileptic drugs

Epilepsy is known as a serious neurological disorder characterized by unpredictable seizures. Epilepsy is affecting more than 60 million people worldwide. Intractable epilepsy (IE), in which a patient's seizures fail to come under control with pharmacotherapeutics, is a severe form of epilepsy, occurring in 20-30% of patients diagnosed with epilepsy. The underlying mechanisms

of IE are not fully clear, although there are several explanations found in the literature. One of these focuses on overexpression of P-gp transporters, MDR transporters, and other efflux transporters at the BBB in the cerebrovascular endothelium around the epileptic focus in the brain (19,78). However, it is not clear whether this upregulation of P-gp is caused by epilepsy, by uncontrolled recurrent seizures, by drug-associated induction of efflux transporters, by genetic polymorphisms or by a combination of all of these factors (79).

Ischemic stroke

During ischemic stroke, the blood flow is locally disrupted leading to deprivation of brain tissue from oxygen and glucose. Subsequently, a lack of sufficient oxygen activates hypoxia-inducible factor 1 alpha (HIF-1 α) and nuclear factor κ B, leading to a time-dependent increase in P-gp expression (80–82). This increase is thought to be an attempt of the ischemic brain to maintain a functional barrier between the blood and the brain when the BBB is compromised (83,84). However, an increase in P-gp function might cause a reduced uptake of therapeutic drugs in the brain after ischemic stroke.

Aging

Since aging is the most important risk factor for the development of AD, and P-gp is a known efflux transporter of A β , there is an increasing interest in the function of P-gp in normal aging and Alzheimer's disease. A β transport at the BBB is significantly altered with increasing age. In 2002, Vogelgesang et al. studied brain tissue samples of 243 non-demented subjects and found there was an age-related decrease in P-gp in association with an increase of amyloid deposition (85). A later PET study with the tracer (*R*)-[¹¹C]verapamil also reported a decrease of P-gp function with increasing aging (86). Chiu et al. showed a trend of reduced P-gp function with advancing age and also a significant reduction in P-gp function in Alzheimer's disease patients compared with healthy elderly controls (87). Amyloid transporter gene expression is age-dependent, which may result in changes in protein expression at the BBB (88). Besides a reduction in P-gp function, a reduction in Lipoprotein Receptor-Related Protein 1 (LRP-1) efflux transporter function and an increase in the function of Receptors for Advanced Glycation Endproducts (RAGE) was found in animal studies. The latter changes may result in an increased influx of A β into the brain (89).

Polymorphism, genotyping and individualized medicine

The ABCB1 gene, which codes for P-gp displays more than 50 single nucleotide polymorphisms (SNPs) (90). Variability in pharmacogenes can influence the metabolism, transport, and elimination of drugs. This makes the variability in pharmacogenes an interesting field of research since it may alter the response of patients to specific pharmaceuticals. Different forms of such genes may be associated with inter-individual variations in drug-efficacy or the severity of unwanted side-effects. Pharmacogenetic data is also of great value in the prediction of neurological diseases. Since ABC transporters influence the uptake of many CNS pharmaceuticals, the question arises whether polymorphisms may predict therapy outcome. The hypothesis that variation in the ABCB1 gene is associated with antidepressant treatment outcome has been tested in various clinical studies, and the results were inconclusive. In a meta-analysis of 16 studies in which the association between ABCB1 polymorphisms and antidepressant treatment outcome was analyzed, Breitenstein et al. found that SNP rs2032583 and rs2235015 have a significant influence on treatment outcome and could possibly be used as predictors of treatment outcome in depression (91). However, despite the extensive number of studies performed, no unambiguous answer to the question of the predictive value of ABCB1 polymorphisms was reached. Multiple studies have found an association between MDR1 C3435T polymorphism and antiepileptic drug resistance (92,93).

Drug resistance and ABC transporters

Pharmaceuticals can cross the BBB and enter the brain via six major pathways: paracellular transport, passive transcellular diffusion, carrier-mediated transport, receptor-mediated transcytosis, adsorptive-mediated transcytosis, and cell-mediated transport. In various diseases including CNS disorders, oncologic diseases, and infectious diseases, the effect of treatment is limited because of drug resistance. A prerequisite for adequate distribution of the drug to its target is adequate transport between various tissues and compartments. Distribution of drugs to the brain is dependent on both the properties of the BBB and the properties of the drug (94,95). An efflux transporter as P-glycoprotein can have a great impact on the distribution of various drugs and is thought to be responsible for drug resistance in cancer, epilepsy, Alzheimer's disease and schizophrenia (96–98). Drug resistance may be due to two underlying causes: First, the drugs used in the treatment of those diseases are substrates of ABC transporters. Second, the expression of the ABC transporters involved in drug efflux can be altered by the disease itself. Studies in the field of drug resistance have led to important new developments, such as specific transport inhibitors, specific antibodies for the transporters and new in vivo imaging techniques (e.g. novel

PET-tracers). However, drug resistance is still a major therapeutic obstacle and further studies are required.

Mechanism of Action of ABC Transporter-Binding Substances

Typically, the ABC transporters consist of a pair of NBDs, present on the membrane side in contact with the cytoplasm and two TMDs. There exist ABC transporters with a different number of TMDs and NBDs. The NBDs are responsible for the ATP binding and hydrolysis and thus they provide the energy for the active transport of substances. The TMDs are the substrate-binding site which contributes to the transport specificity (99).

It was initially thought that the P-gp transporter possessed two binding sites (100). Then, a third ligand-binding site was discovered (101). And later on, Martin et al suggested that the P-gp transporter contained four ligand-binding sites (102). Sites I, II and III work as transport sites because they interact with substrates (such as paclitaxel, vinblastine) and modulators (tariquidar). Site IV, on the other hand, acts as a regulatory site. Since a wide number of compounds can also interact with BCRP and MRP1, these transporters may also have several ligand-binding sites similar to the P-gp transporter (103,104).

The compounds that interact with the ABC transporters can behave as substrates, inhibitors, inducers or activators. Substrates are pumped out of the cell in an ATP-dependent manner, inhibitors reduce the ABC transporter function, inducers increase the transporter expression and activators enhance the transporter function. Notice that one compound can have different modes of action (depending on the administered dose): drugs that are P-gp substrates can also act as P-gp inhibitors or inducers (105). It is important to mention that induction and activation are not always related. A compound can increase the expression level of the transporter but not increase its functionality and vice versa (103).

Different hypotheses try to explain how inhibitors interfere with the transporter. The inhibitor may act by blocking the ligand-binding site, by preventing the ATP hydrolysis or by modifying the integrity of the lipid membrane. There are different types of inhibitors, classified by their potency and selectivity (106). Some P-gp inhibitors are used as therapeutic agents and are co-administrated with chemotherapeutic agents or anti-epileptic drugs to overcome drug resistance in cancer or epilepsy (105).

An inducer promotes upregulation of the protein expression (103). Many structurally unrelated compounds have been reported as P-gp inducers such as abacavir, aldosterone, hypericin, morphine, and rifampicin (106). Numerous routes can regulate the transcription of the MDR1 (ABCB1) gene, which highlights the ability of P-gp to respond to a wide variety of stimuli. Upregulation of the MDR1 gene depends on different transcription factors (pregnane X receptor (PXR), heat-shock transcription factor 1 (HSF-1); nuclear factor Y (NF-Y); early growth response protein 1 (EGR-1)) that do not bind directly to P-gp (106). The nuclear factor PXR can be activated by diverse compounds such as rifampicin, phenobarbital, and mifepristone. PXR upregulates the MDR1 expression, which subsequently leads to an increased expression of P-gp (105,107).

Activators bind to the protein and induce a conformational change that enhances the transport of other substrates bound to a different ligand-binding site (103). These compounds increase the P-gp function without increasing the protein expression. Therefore, activators induce the P-gp function as long as they are present. This is a more rapid mechanism than the action of P-gp inducers (106).

Since many compounds can modify the function of the ABC transporters (in particular P-gp and BCRP), the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) developed guidelines for the screening of drugs that cause transporter-mediated drug-drug interactions (DDI) (104,108,109). Concomitant administration of drugs that can interfere with ABC transporters may cause alterations of drug concentrations and therefore, may lead to decreases in drug efficacy or to toxicity. Appropriate measures should be taken to prevent the clinical implications of transport-mediated DDI. Treatment effects should be monitored, administered dose should be adjusted if necessary, and alternative therapies may have to be sought (110).

Use of P-gp Modulators to Treat Drug Resistance, Neurological Disorders and other Conditions.

Since P-gp dysfunctions are encountered in many different diseases of the CNS, P-gp modulators may restore the proper function and may improve the disease condition.

P-gp Inhibitors and Drug resistance

Resistance to drug treatment is a common clinical issue in cancer and infectious diseases and it also occurs in rheumatoid arthritis, epilepsy, depression and other psychiatric disorders. The role of P-gp and other ABC transporters in drug resistance has gained increasing attention (111).

P-gp inhibitors have been developed and used to overcome drug resistance in a wide variety of diseases. P-gp inhibitors can be classified into four generations (106,112). The first generation of P-gp inhibitors includes verapamil and cyclosporine A. These inhibitors are characterized by their low therapeutic response and high toxicity (cardiovascular toxicity). The second generation (e.g. valsopodar) was developed in order to achieve higher potency and reduced toxicity. However, these compounds also inhibit cytochrome P450 and many DDIs were encountered when the inhibitors were co-administrated with chemotherapeutic agents. Thus, a third generation of P-gp inhibitors was developed. These compounds were 200-fold more potent than the first and second generation of inhibitors (112). Some of these inhibitors (e.g., tariquidar) were evaluated in clinical trials (112,113). Recently, natural substances have been proposed to reverse drug resistance. These compounds, which represent the fourth generation of P-gp inhibitors (106), are obtained from different natural sources. They include alkaloids, flavonoids, coumarins, resins, etc. and are characterized by low cytotoxicity and increased oral bioavailability (112,114).

In cancer drug resistance, it has been suggested that the overexpression of efflux transporters in tumors reduces the drug concentration in the target tissue and consequently limits the treatment efficacy (115,116). P-gp, MRP1, and BCRP are the most relevant efflux transporters related to drug resistance. A wide variety of anticancer drugs such as vinka alkaloids (vincristine), taxanes (docetaxel and paclitaxel), anthracyclines (doxorubicin), topoisomerase inhibitors such as topotecan, and tyrosine kinase inhibitors (gefitinib) are substrates of the P-gp transporter (117).

To date, clinical trials with P-gp inhibitors in combination with anticancer drugs were unsuccessful. Several causes may explain the failures. First, the low affinity of early-generation inhibitors required the use of high drug doses which led to severe side effects and toxicity. Moreover, the concomitant administration of P-gp inhibitors and anticancer drugs, reduces the metabolism and excretion of the chemotherapeutic agents, and increases their concentration in plasma (117). Contradictory conclusions were obtained with regards to tariquidar. Some studies identified tariquidar as a P-gp inhibitor and P-gp substrate depending on the used dose. Others reported that tariquidar can also act as a BCRP inhibitor and substrate (118). The structure of the binding pocket of the P-gp transporter is not well-known, which precludes the development of potent and highly specific P-gp inhibitors (117).

The P-gp transporter located at the BBB also decreases the concentration of numerous unrelated drugs in the CNS. For instance, it has been shown that antiepileptic drugs such as phenytoin, carbamazepine, phenobarbital, lamotrigine, and gabapentin are substrates of P-gp (19). Therefore, it has been suggested that P-gp plays an important role in drug-resistant epilepsy or IE (19,119).

Many scientific publications support the role of P-gp in drug-resistant epilepsy. Preclinical studies have found that phenobarbital resistant rats showed higher P-gp expression levels at the BBB than phenobarbital responsive rats (120). Moreover, chronic administration of phenobarbital caused a reduction of phenobarbital concentration in the hippocampus and an increase of P-gp expression (121). Despite the unsuccessful strategy of using P-gp inhibitors to overcome cancer drug resistance, it has been proposed that the co-administration of P-gp inhibitors and antiepileptic drugs could restore the antiepileptic efficacy. In support of this idea, the administration of tariquidar together with phenobarbital to phenobarbital-resistant rats counteracted the phenobarbital resistance (122). Moreover, the co-administration of verapamil and carbamazepine to patients with IE improved the efficacy of the antiepileptic drug (20). Therefore, modulation of the P-gp function using P-gp inhibitors may be a new approach to overcome drug resistance in epilepsy.

The use of P-gp inhibitors in treatment-resistant depression has also been suggested. Around 60% of patients with depression do not show a satisfactory response to their medication and again the P-gp transporter may play a role in limiting the brain penetration of the applied antidepressant drugs (123). In vivo studies performed in mice demonstrated that co-administration of the P-gp inhibitor verapamil and the antidepressant escitalopram for 22 days increased the brain concentration of the antidepressant (124). However, further studies are needed to elucidate the relationship between P-gp function and treatment-resistant depression.

New strategies to overcome drug resistance in cancer and other pathologies have been explored such as the development of drugs that do not show affinity to the ABC transporters and the use of advanced delivery systems such as liposomes or gold nanoparticles as well as the use of antibody-drug conjugates. These new approaches aim to bypass the P-gp efflux action, to target the drug delivery to a specific tissue and to reduce the toxic side effects of the drug (111,112,117).

P-gp Inducers and Activators to Treat Neurodegenerative Disease and Intoxications

P-gp inducers and activators (such as St. John's wort (*Hypericum perforatum*), dexamethasone, vinblastine, and rifampicin (106)) increase P-gp function and/or expression and they may be used to reduce the brain penetration of harmful substances (103). The use of P-gp inducers or activators can be important in the treatment of intoxications (125,126). For instance, the administration of dexamethasone to paraquat-exposed rats decreased paraquat accumulation in the lungs and reduced the lung damage (126).

Another potential use of inducers and activators was suggested by Haslam et al., based on the finding that the upregulation of ABC transporters in the hair follicle prevents alopecia induced by chemotherapy. This strategy may reduce hair loss in cancer patients (127,128).

Additionally, inducers have been proposed as a potential treatment for Alzheimer's and Parkinson's disease. In the case of Alzheimer's disease, A β has been identified as a substrate of the P-gp transporter (10). Vogelgesang et al. found an inverse correlation between the deposition of A β in the brain of elderly humans and the P-gp expression in endothelial cells of the cerebral vasculature (129). Recent research also highlighted the relationship between the MDR1 gene, which is encoding P-gp, and the risk of developing Parkinson's disease (73). It has been suggested that repeated contact with pesticides is associated with the development of Parkinson's disease in patients carrying a c3435T polymorphism of the MDR1 gene (14,130,131). Furthermore, PET studies have indicated that the P-gp function is decreased in Alzheimer's and Parkinson's disease (14,132), thus the use of P-gp inducers may help to treat the disease.

Some preclinical studies have already demonstrated that P-gp inducers such as pregnenolone-16 α -carbonitrile can restore the P-gp function at the BBB and reduce the A β deposition in the brain (133). Similar results were obtained in mice models of Alzheimer's disease after treatment with St. John's wort. Animals receiving St. John's wort showed a reduction of A β accumulation and an increase in the cerebrovascular expression of P-gp (134).

Many efforts have been made to find new molecules able to induce or activate the P-gp function, which could be applied to treat intoxication or neurodegenerative disease. Recently, MC111 was identified as a promising candidate, due to its capacity to induce P-gp and BCRP expression in SW480 human colon adenocarcinoma cells (135). In vivo studies using rats and the PET tracer [^{18}F]MC225 are ongoing to confirm these findings.

PET

Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) together with magnetic resonance imaging (MRI) and computed tomography (CT) are imaging techniques that allow noninvasive tracking of pathophysiological processes in various neuropsychiatric disorders. Molecular imaging has also been used in various aspects of drug development (136). For an efficient visualization, characterization, and measurement of biological processes at the molecular, cellular, whole organ, or body level, specific imaging probes are needed. These probes are also known as tracers and provide functional information, like the magnitude of regional blood flow, glucose metabolism, or receptor density which can be

presented either as a two- or three-dimensional image. PET is the most sensitive technique for in vivo imaging and quantification of biological and pathophysiological processes. However, the spatial resolution of PET is relatively low, and the additional use of CT or MRI imaging is required to provide high-resolution structural images that can be fused with the functional PET image. PET/CT or PET/MRI scanners provide both anatomical and functional information in a single scanning session.

The decay of radionuclides for PET imaging occurs by the emission of a positively charged particle called positron. When emitted from the nucleus, the positron travels a very short distance (positron range, 3–5 mm) in the surrounding tissue before it annihilates by combining with an electron. The mass of the positron and electron is then converted to energy resulting in two 511 keV γ -photons which are emitted simultaneously at an angle of approximately 180°. The pair of photons is detected by rings of detectors in the PET scanner. The acquisition of a large number of coincident events provides data that can be reconstructed to an image with information on the spatial distribution of radioactivity as a function of time.

Traditionally, PET tracers for imaging of ABC transporters are evaluated using a dual-scan protocol. This protocol involves a baseline PET scan where the efflux transporters are working adequately and an after-treatment scan where the function of these transporters is either inhibited or induced. An inhibitor is usually used for the challenge, which will reduce the transporter function and therefore, increase the brain uptake of the tracer (*137,138*). By contrast, if an inducer is used the cerebral uptake of the tracer will decrease. Since the majority of the ABC transporter tracers behave as strong transporter substrates, their baseline uptake of radioactivity in the brain is very low, thus measuring decreases of this uptake is complicated. For this reason, inhibitors are commonly used to validate PET tracers for ABC transporters. Once, an appropriate and specific tracer is found, PET imaging allows to assess the function of that particular ABC transporter in the healthy or diseased human brain and to evaluate the impact of treatments on the function of this transporter.

Recently, the introduction of whole-body PET, which allows simultaneous assessment of tracer uptake in all organs and tissues of the human body, has roused interest in studying the connection among different systems that may play a role in a particular disease (*139*). Using this technique, it may, for instance, be possible to study the role of the gut-brain axis in CNS diseases. It has been pointed out that the microbiota present in the gastrointestinal tract has an important impact on the brain. The gut flora not only affects the enteric nervous system but also the CNS via neuroendocrine and metabolic pathways (*140*). Changes in the microbiota are related to anxiety and depression (*141*). The onset of other brain disorders such as schizophrenia, autism and

Parkinson's disease has also been related to dysfunction of the gut-brain axis (142). Recent publications suggest that the gut microbiota can affect the integrity of the BBB and may contribute to the development or the progression of neurodegenerative diseases (143). The microbiome of Alzheimer's disease patients may also cause a reduction of P-glycoprotein expression in the intestinal epithelial cells (144). Dynamic whole-body PET may bring the opportunity to explore and compare the functionality of the P-gp transporters at the BBB and the intestinal tract. This new approach may broaden insight in the gut-brain axis and its implication in the onset of neurodegenerative diseases.

PET Tracers for Imaging of ABC Transporter Function and Expression in the BBB: Background

The function of the ABC transporters can be assessed by Positron Emission Tomography. Since PET is a non-invasive technique, it allows studying the same subject at different time points e.g. before and after treatment, or at different stages of a disease. Moreover, since longitudinal studies can be performed, the number of subjects required to reach statistical significance is reduced (136). Specific tracers should be designed in order to explore the function of ABC transporters.

In 1998, Hendrikse et al. used [^{11}C]verapamil to image the P-gp function for the first time (145). Since then, many other compounds have been radiolabeled with carbon-11 or fluorine-18 to explore the function of P-gp and other ABC transporters at the BBB (146): (*R*)-[^{11}C]verapamil (147), [^{11}C]-N-desmethyl-loperamide ([^{11}C]dLop) (148), [^{11}C]phenytoin (149), [^{18}F]paclitaxel (150), [^{11}C]laniquidar (151), [^{11}C]tariquidar (152), [^{18}F]MC225 (153) and [^{11}C]metoclopramide (154). Although numerous research studies have been performed with these tracers, none of the tracers was incorporated into the routine medical practice.

The majority of the ABC transporter tracers have been aimed at the imaging of P-gp. Several attempts have been made to develop PET tracers for other ABC transporters, with limited success. Most BCRP and MRP1 substrates proved to show also affinity towards the P-gp transporter. Because of this lack of specificity, the function of a particular transporter cannot be distinguished from the action of other systems (146,155).

PET studies with "BCRP-selective compounds" did not show differences of uptake in wild-type and BCRP knockout mice or rats (156–158). Since BCRP is the most abundant ABC transporter in the human brain, Wanek et al. designed a protocol for visualizing this transporter at the BBB. The protocol consists of using the dual P-gp/BCRP tracer [^{11}C]tariquidar in combination with cold tariquidar or in P-gp knockout mice. P-gp function is then inhibited or absent and therefore, the function of BCRP can be assessed (159).

Two PET tracers have been developed for the MRP1 transporter: 6-bromo-7- $^{[11}\text{C}]$ methylpurine (160) and 6-bromo-7-(2- $^{[18}\text{F}]$ fluoroethyl)purine (161). Both are pro-drugs that after entering the brain are conjugated with glutathione and as a result, become substrates for MRP1. In vivo experiments showed significant differences in the brain clearance of both tracers between wild-type and MRP1 knockout mice (160,161). Since a relationship between the MRP1 transporter and A β clearance from the brain has been proposed, additional studies were performed in a beta-amyloidosis mouse model (APP/PSI-21) using 6-bromo-7- $^{[11}\text{C}]$ methylpurine ($^{[11}\text{C}]$ BMP). The study evaluated the MRP1 function in APP/PSI-21 mice and in wild-type mice. The study revealed a significantly higher value of the elimination constant (k_{elim}) after the treatment with the MRP1 inhibitor MK571 in the APP/PSI-21 mice compared to the wild type mice, which represents an increase of MRP1 expression in the astrocytes. Moreover, western blot results showed an increase in MRP1 levels which was positively correlated with the induction of the MRP1 activity (162). This study suggests that $^{[11}\text{C}]$ BMP may be used to measure the astrocyte MRP1 function (162). Unfortunately, species differences in the expression levels of MRP1 hamper the translation of preclinical data to humans (163).

To date, the most commonly used PET tracers for imaging P-gp function at the BBB are (*R*)- $^{[11}\text{C}]$ verapamil and $^{[11}\text{C}]$ dLop. Although these tracers have been extensively used in small animals and humans, both tracers have some important drawbacks (155). They act as strong substrates of the P-gp transporter which means that the tracers have a high affinity for the transporter protein. Consequently, the tracers are pumped out from the brain to the blood with very high efficiency and low uptake of the tracer is observed at baseline conditions, when the transporter is fully functional (164,165). Due to the low uptake at baseline, these PET tracers are not able to measure increases in the P-gp function as occur in drug-resistant epilepsy (19). However, (*R*)- $^{[11}\text{C}]$ verapamil and $^{[11}\text{C}]$ dLop can detect decreases in the P-gp function. Such decreases will result in higher uptake of radioactivity in the brain. Thus, these tracers can be used to study diseases characterized by a decline in P-gp function, such as Alzheimer's disease (166).

Another important disadvantage of (*R*)- $^{[11}\text{C}]$ verapamil is its extensive in vivo metabolism resulting in the formation of radioactive metabolites (dealkylation fragments and polar metabolites) that can cross the BBB. Some of these metabolites are also P-gp substrates (167). A PET study radiolabeled the main metabolite of (*R*)- $^{[11}\text{C}]$ verapamil, called D617 (168). This study evaluated the kinetics of $^{[11}\text{C}]$ D617 to investigate whether the kinetics of (*R*)- $^{[11}\text{C}]$ verapamil and $^{[11}\text{C}]$ D617 are similar and $^{[11}\text{C}]$ D617 can be used as an alternative tracer to measure the P-gp function (168). However, although the kinetics of $^{[11}\text{C}]$ D617 were comparable to that of (*R*)- $^{[11}\text{C}]$ verapamil at baseline, the increase of the brain uptake of $^{[11}\text{C}]$ D617 after P-gp inhibition was

lower. A similar approach was more successful in the case of loperamide. [^{11}C]dLop which is the main metabolite of [^{11}C]loperamide became the preferred P-gp tracer, as its imaging data were more easily interpreted than those of the parent compound (169).

In the case of (*R*)-[^{11}C]verapamil, the presence of radioactive metabolites can lead to quantification errors. Even though the radioactivity in blood samples can be corrected for the metabolite fraction, the radioactivity in tissue cannot be corrected and metabolites will contribute to the tissue signal (155,156). This issue can be solved by using short PET acquisition times because metabolism is negligible shortly after radiotracer injection (170).

Radiolabeling with carbon-11 has the disadvantage of a short physical half-life of the radiotracer (20.4 minutes) and a low spatial resolution of PET images (because of the long-range of the ^{11}C positron ($^{+}\beta$)). The use of fluorine-18 for radiolabeling allows the distribution of the resulting PET tracer to remote imaging centers and allows using longer scan times because of the longer physical half-life of ^{18}F (109.8 minutes) (165,171).

Since the perfect tracer to measure the P-gp function does not yet exist, new tracers for ABC transporters are needed. Ideal PET tracers should be produced in high overall yields and with high molar activities. They should display a lipophilicity above 2 and below 3.5 to ensure adequate brain penetration and limited non-specific binding in the brain (172). Moreover, these molecules should show a high specificity for the desired ABC transporter. They should be slowly metabolized and their metabolites should not cross the BBB and not be substrates for ABC transporters. The parent tracers should show a moderate affinity to the ABC transporter of interest, resulting in a moderate brain uptake at baseline. They would then be able to measure decreases as well as increases of the transporter function at the BBB. Radiolabeling should be done with fluorine-18, to obtain high-quality images (138,155,165).

Many strategies have been explored to overcome a low baseline uptake of radioactivity in the brain. The first approach was to develop PET tracers with greater lipophilicity to enhance their passive diffusion to the brain, however, high lipophilicity may lead to increased non-specific binding (137,173). Next, well-known P-gp inhibitors such as tariquidar, elacridar, and laniquidar were radiolabeled with carbon-11 to obtain radiotracers that would bind to P-gp but not be transported. In theory, this approach would provide a higher brain uptake at baseline and would allow measurement of P-gp expression in the brain. Unfortunately, at low concentrations, these inhibitors were recognized as substrates by P-gp and BCRP at the BBB of rats. Therefore, these tracers were transported out of the brain, resulting in a low baseline uptake. This strategy of inhibitor labeling proved to be unsuccessful (155). A third approach to increase the baseline

uptake of PET tracers for P-gp consists of the development of lower avidity substrates. This approach using weak P-gp substrates aimed at higher baseline uptake in the brain which facilitates the measurement of increases in the P-gp function. Several radiotracers have already been identified as weak P-gp substrates and their kinetics are being evaluated (149,154,174,175).

Recently, it has been noticed that (*R*)-[^{11}C]verapamil and [^{11}C]dLop possess limited sensitivity to detect small changes in the P-gp function. The brain distribution of (*R*)-[^{11}C]verapamil and [^{11}C]dLop was not significantly increased in heterozygous mice (+/-), which have 50% lower P-gp levels, compared to wild-type mice. These results indicate that the P-gp function should be reduced by more than 50% to effectively limit the entrance of P-gp substrates in the brain (176). The limited sensitivity of the existing PET tracers may be due to their high affinity to the transporter. It has been proposed that PET imaging with weak P-gp substrates may result in higher sensitivity for detecting small variations in the P-gp function, as may occur in several diseases (164,177).

Substrates as Tracers for Measuring P-gp Function

Strong Substrates (figure 2)

Figure 2: Structures of strong P-gp substrates: (*R*)-[^{11}C]verapamil (A), [^{11}C]dLop (B), [(*R*)-N-[^{18}F]fluoroethylverapamil (C), (*R*)-O-[^{18}F]fluoroethylnorverapamil (D) and [^{18}F]MPPF (E).

[¹¹C]verapamil

The L-type calcium channel blocker verapamil [2,8-bis(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaocitanitrile] is widely used in the treatment of hypertension (178), angina pectoris, and cardiac arrhythmias. Verapamil metabolizes preferentially to norverapamil via N-demethylation (179). After oral administration, the (*S*)-enantiomer of verapamil is metabolized more actively than (*R*)-verapamil in humans, resulting in a 2.5 times higher concentration of the (*R*)-enantiomer in plasma (180). (*R*)-verapamil has only 5–10% of the calcium channel blocking activity of the (*S*)-enantiomer. However, the (*R*)-enantiomer is equipotent to racemic verapamil and (*S*)-verapamil in its efficacy to inhibit P-gp. Verapamil is metabolized by cytochrome P450 enzymes and some of the radioactive metabolites are themselves substrates and inhibitors of P-gp (181). In addition, polar radiolabeled metabolites are formed, which result in a non-P-gp-mediated signal (167). Quantification of racemic [¹¹C]verapamil is difficult because the enantiomers have different pharmacokinetic properties (182). Therefore, PET studies need to be performed with an optically pure enantiomer. (*R*)- and (*S*)-[¹¹C]verapamil were prepared from (*R*)- and (*S*)-desmethylverapamil by methylation with no-carrier added [¹¹C]methyl iodide or [¹¹C]methyl triflate (183). Although no difference in P-gp mediated transport selectivity between (*R*)- and (*S*)-verapamil was found (184), (*R*)-[¹¹C]verapamil was selected as the most appropriate tracer for measuring P-gp function with PET because the (*R*)-enantiomer was less metabolized in humans and showed a lower affinity for calcium channels. This tracer showed a low brain uptake at baseline both in humans and other vertebrate species, but coadministration with P-gp inhibitors as cyclosporin A (CsA) or tariquidar increased its volume of distribution (V_T) and kinetic constant k_1 (185,186). The increase in brain V_T after administration of tariquidar was approximately +300 % in rats and $+24 \pm 15$ % in humans (187,188), which points to a pronounced species difference regarding the tariquidar effect.

[¹¹C]N-desmethyl-loperamide

Loperamide is an antidiarrheal agent that acts through agonism at μ -opioid receptors in the gut (189). This drug is normally without harmful central effects because P-gp expels it from the brain. [¹¹C]loperamide is avidly metabolized to [¹¹C]dLop via demethylation (190). This metabolite is also a substrate for P-gp and it has been used as a PET tracer instead of [¹¹C]loperamide. Demethylation of [¹¹C]dLop may occur, but it will lead only to the formation of single-carbon radiometabolites such as [¹¹C]methanol. These metabolites will be oxidized and ultimately expired as [¹¹C]carbon dioxide. They should not accumulate in brain tissue and will therefore not cause problems in the kinetic analysis of the acquired PET data. The uptake of [¹¹C]dLop 30 minutes after its injection was about 3.5-fold higher in the forebrain of P-gp knockout mice

compared to wild-type mice supporting the results from previous studies which indicate that [^{11}C]dLop is a substrate of P-gp (169). Similar results were acquired in monkeys. [^{11}C]dLop was found to be selective for P-gp when tested in human cell lines that overexpress P-gp, MRP1, or BCRP and also in P-gp knockout mice (191). At low concentrations (≤ 1 nM) dLop acts only as a substrate, but at higher concentrations (≥ 20 μM) it acts both as a substrate and an inhibitor. Because low concentrations of radiotracer are used in PET imaging, [^{11}C]dLop will act only as a selective substrate for P-gp at the BBB. Pretreatment with the inhibitor tariquidar increases the brain uptake of [^{11}C]dLop markedly in monkeys and humans (192). After entry of [^{11}C]dLop into the brain, it is trapped and does not wash out. Although dLop is an opiate agonist, its trapping is not a result of high-affinity binding to the opiate receptor. Brain uptake of [^{11}C]dLop cannot be displaced by receptor-saturating doses of an opiate agonist or antagonist (169). This irreversible trapping amplifies the PET signal so it is advantageous from an imaging perspective. dLop has a pK_a of 7.3 and may be trapped by passive diffusion into acidic organelles, mainly lysosomes, followed by protonation within the organelle (193,194). To test this hypothesis the accumulation of [^3H]dLop was measured in human cell lines and compared to the uptake of other weak bases (195). [^{11}C]dLop was found to be ionically trapped in acidic lysosomes and tariquidar competed for lysosomal accumulation, both in vitro and in vivo. This interaction is problematic in peripheral organs but not in the brain. Thus [^{11}C]dLop can still be used, either alone or in combination with tariquidar, to measure the function of P-gp at the BBB. However, the low brain uptake of [^{11}C]dLop at baseline conditions complicates the evaluation of increases in the P-gp function, as is also the case with (*R*)-[^{11}C]verapamil.

Fluorine-18 verapamil analogs

The synthesis of two fluorine-18 (*R*)-verapamil analogs has also been reported: (*R*)-N-[^{18}F]fluoroethylverapamil and (*R*)-O-[^{18}F]fluoroethylnorverapamil. Since (*R*)-[^{11}C]verapamil is considered as the gold standard for measuring the P-gp function until now, these two candidates were proposed as promising radiofluorinated analogs. (*R*)-N-[^{18}F]fluoroethylverapamil was obtained as follows: first, [^{18}F]fluoroethyl triflate was obtained from bromoethyltosylate using a silver triflate column and secondly, [^{18}F]fluoroethyl triflate was distilled into a vial containing the precursor (*R*)-norverapamil. The synthesis of (*R*)-O-[^{18}F]fluoroethylnorverapamil was simpler. The precursor contained a tosyl group which allows direct fluorination, but Boc-deprotection was also required. Preclinical studies carried out in rats showed that after administration of the P-gp inhibitor tariquidar, the brain uptake of (*R*)-N-[^{18}F]fluoroethylverapamil and (*R*)-O-[^{18}F]fluoroethylnorverapamil was increased by 3.6- and 2.4-fold respectively. A PET study using P-gp knockout and P-gp and BCRP double knockout mice showed that (*R*)-O-[^{18}F]fluoroethylnorverapamil was more specific for P-gp than (*R*)-N-[^{18}F]fluoroethylverapamil; thus, this analog was selected as the best tracer candidate (171).

[¹⁸F]MPPF

The radiosynthesis of 4-(2'-methoxyphenyl)-1-[2'-(N-2"-pyridinyl)-p-[¹⁸F]fluorobenzamido]ethylpiperazine ([¹⁸F]MPPF) comprises nucleophilic aromatic substitution of a para-nitro compound. The synthesis was performed in the presence of K₂CO₃ and Kryptofix₂₂₂ and under microwave heating (196). [¹⁸F]MPPF is a PET tracer aimed at the imaging of the serotonin 5-HT_{1A} receptor, however, it was discovered to be also a substrate of P-gp. Administration of the P-gp inhibitor cyclosporine A increased the tracer uptake in rat hippocampus (197). This observation was confirmed by biodistribution studies performed in P-gp knockout mice. The tracer uptake in the brain of P-gp knockout animals was 2 to 3-fold higher than in wild-type mice (198). However, in cells transfected with human P-gp or BCRP genes, [¹⁸F]MPPF efflux was not reduced by the P-gp inhibitor valsopodar, and thus, [¹⁸F]MPPF may not be transported by the human P-gp. A study performed in non-human primates showed an increase in the uptake of [¹⁸F]MPPF in the brain after the treatment with cyclosporine, however, this increase was attributable to an increase of the free fraction of the tracer in plasma. This study noted that cyclosporine A binds to plasma proteins and displaces other compounds (such as [¹⁸F]MPPF) from its plasma protein binding sites (199). All these findings restrict the use of [¹⁸F]MPPF as a PET tracer for measuring the function of P-gp at the BBB.

Weak P-gp Substrates (figure 3)

Figure 3: Structure of weak P-gp substrates: [¹¹C]Phenytoin (A), (R)-[¹¹C]Emopamil (B), [¹⁸F]MC225 (C) and [¹¹C]metoclopramide (D).

[¹¹C]Phenytoin

In 2010, an in vitro study showed that phenytoin and phenobarbital (antiepileptic drugs) are transported by the human P-gp, which strengthens the hypothesis that P-gp is involved in antiepileptic drug resistance (200). In rhesus monkeys [¹¹C]phenytoin showed a high brain uptake (201), which suggests that phenytoin behaves as a weak P-gp substrate (149). [¹¹C]phenytoin was synthesized via [¹¹C]CO using a high-pressure autoclave (149). In rats, the baseline uptake of [¹¹C]phenytoin was higher than that of [¹¹C]verapamil. After administration of 15 mg/kg of tariquidar, the brain uptake of [¹¹C]phenytoin showed a smaller increase compared to avid substrates tracers such as [¹¹C]verapamil. This observation also indicates a moderate affinity of [¹¹C]phenytoin to P-gp (149). Quantification of the brain uptake of [¹¹C]phenytoin in humans was assessed by different kinetic models. The results of this study indicated that the 1-tissue compartment model (1-TCM) is the preferred technique to analyze the data and K₁ and V_T can be used for measuring the P-gp function (202). Although [¹¹C]phenytoin seems to be a good tracer candidate to measure increases in the function of P-gp, further studies performed under pathologic conditions are still required.

[¹¹C]Emopamil

2-Isopropyl-5-[methyl-(2-phenylethyl)amino]-2-phenylpentanenitrile (emopamil) is a calcium channel blocker. Its chemical structure is similar to verapamil. A study evaluated the brain penetration of emopamil, verapamil, and gallopamil in rats and concluded that the cerebral availability and BBB permeability was higher for emopamil than for verapamil and gallopamil (203). The rather high brain penetration of emopamil at baseline condition encouraged the development of [¹¹C]emopamil as a weak substrate tracer for P-gp (174). (*R*)- and (*S*)-[¹¹C]emopamil were synthesized via methylation of the precursors, (*R*)- and (*S*)-noremopamil, with [¹¹C]methyl triflate. From mice experiments, it was concluded that (*R*)-[¹¹C]emopamil provides higher uptake than (*S*)-[¹¹C]emopamil. (*R*)-[¹¹C]emopamil showed a 2-fold higher area-under-the-curve (AUC) of the brain radioactivity in baseline scans than (*R*)-[¹¹C]verapamil. However, after the administration of cyclosporine A, the AUCs of the brain radioactivity of both tracers were similar. Unfortunately, both (*R*)- and (*S*)-[¹¹C]emopamil are rapidly metabolized (174). To verify that (*R*)-[¹¹C]emopamil is suitable for measuring P-gp upregulation, additional experiments in animal models are needed.

[¹⁸F]MC225

Savolainen et al. evaluated three fluorine-18 compounds that shared the same moiety: 6,7-dimethoxytetrahydroisoquinoline, which is also present in tariquidar and elacridar (153). The compounds were assessed in vitro and in vivo with PET imaging, using P-gp and BCRP knockout

mice. The results were compared to those obtained with [^{11}C]verapamil. From this experiment, [^{18}F]MC225 was selected as the most appropriate candidate to measure P-gp function at the BBB. The synthesis of [^{18}F]MC225 consists of two steps. First, [^{18}F]bromoethylfluorine is synthesized from 2-bromoethyltosylate by heating at 90°C for 15 minutes in dichlorobenzene. Afterward, the distilled [^{18}F]bromoethylfluorine is trapped in a vial containing sodium hydride and the phenol precursor of MC225 in dimethylformamide (DMF), and the vial is heated at 80°C for 5 minutes. PET studies demonstrated that the baseline uptake of [^{18}F]MC225 was higher than that of [^{11}C]verapamil in rats. Baseline V_T values of [^{11}C]Verapamil ranged from 1.1 to 2.3 (187) whereas values for [^{18}F]MC225 varied from 6.6 to 11 (175). The brain uptake of [^{18}F]MC225 was also higher in P-gp and BCRP knockout mice than in the wild-type, and in addition, [^{18}F]MC225 showed greater metabolic stability (153). [^{18}F]MC225 showed specificity for P-gp over BCRP or MRP1 (153,204). Rat experiments confirmed that [^{18}F]MC225 is transported by P-gp and not by BCRP. Inhibition of P-gp increased the brain uptake of the tracer but inhibition of BCRP did not affect this uptake (175). At 30 minutes after tracer injection, only 24% of the plasma radioactivity represented the parent compound. Different kinetic models were fitted to the data and it was concluded that a 1-TCM was the preferred model for [^{18}F]MC225 in rats. After inhibition of P-gp, the changes in the influx rate constant K_1 were higher than the changes in V_T , for this reason, K_1 was proposed as the best parameter to measure the P-gp function (175), as has also been suggested for analysis of the data of (*R*)-[^{11}C]verapamil (186). PET studies with [^{18}F]MC225 are ongoing to demonstrate that this tracer is able to measure increases and decreases of the P-gp function at the BBB.

[^{11}C]metoclopramide

Metoclopramide is a dopamine D2 receptor antagonist that is used as antiemetic. The drug was not identified as a P-gp substrate during in vitro experiments, however, in vivo studies in mice revealed increased (6.6-fold) brain concentration of metoclopramide in P-gp knockout compared to wild-type mice (205). Patients treated with metoclopramide reported CNS effects, which suggests that metoclopramide crosses the BBB (154). Therefore, [^{11}C]metoclopramide was synthesized by radiomethylation of the methoxy phenol moiety of nor-metoclopramide using [^{11}C]methyl triflate (154). In vitro assays using P-gp and BCRP overexpressing cells demonstrated that [^{11}C]metoclopramide is specific for P-gp over BCRP. Moreover, the brain uptake of the tracer in rats increases after P-gp inhibition, resulting in an increase of K_1 and the binding potential (BP) in the 2-tissue compartment model (2-TCM). At 30 minutes after tracer injection, only 20% of plasma radioactivity represented the parent compound in rats (154). The kinetics of [^{11}C]metoclopramide were also evaluated in non-human primates; P-gp inhibition increased the brain distribution in these animals. In this case, a 1-TCM was used to fit the data, and the most sensitive parameter was the V_T which increased by 2.02-fold. The inhibition also

caused an increase in K_1 (1.28-fold) and a decrease in k_2 (1.64-fold). However, the metabolism of [^{11}C]metoclopramide in non-human primates was more rapid than in rats, and 30 minutes after tracer injection the fraction of the parent [^{11}C]metoclopramide in plasma was below the limit of detection (206). Recently, the kinetics of this tracer was also assessed in humans. Here, the kinetic analysis showed a significant increase in V_T by $29 \pm 17\%$ after P-gp inhibition, mainly due to a significant $15 \pm 5\%$ decrease in k_2 . K_1 was slightly increased after the treatment, however, this increase was significant in only a few regions compared to the baseline scan (207). Further studies in pharmacoresistant patients are needed to demonstrate that [^{11}C]metoclopramide can visualize the increase of the P-gp function in these patients.

P-gp Inhibitors as Tracers for Measuring P-gp Expression

Figure 4: Structure of P-gp inhibitors: [^{11}C]elacridar (A), [^{11}C]tariquidar (B) and [^{11}C]laniquidar (C).

[^{11}C]elacridar, [^{11}C]tariquidar and [^{11}C]laniquidar

Many well-known P-gp inhibitors such as elacridar, tariquidar, and laniquidar were radiolabeled with carbon-11 (figure 4) and later also with fluorine-18. Elacridar, tariquidar, and laniquidar belong to the third generation of P-gp inhibitors. Tariquidar is described as a non-competitive P-gp inhibitor which shows good selectivity toward the P-gp transporter over other efflux transporters, although it also inhibits BCRP transporter but with less potency. Elacridar is

considered as a P-gp and BCRP inhibitor. Laniquidar is a noncompetitive inhibitor less potent than tariquidar and elacridar and is highly selective for P-gp (208). Preclinical studies performed in rats showed that elacridar and tariquidar can increase the uptake of [^{11}C]verapamil in the brain up to 11-fold, with half-maximum effective doses of 1.2 ± 0.1 and 3.0 ± 0.2 mg/kg, respectively. The results also showed that elacridar is three times more potent than tariquidar (209).

For the [^{11}C]elacridar synthesis, the O-desmethyl-elacridar was reacted with [^{11}C]methyl triflate in acetone containing NaOH. [^{11}C]elacridar was administered to mice and rats to evaluate its kinetics. The brain uptake in P-gp knockout mice was 2.5-fold higher and in BCRP knockout mice was 1.3-fold higher compared to wild-type mice. The coadministration of [^{11}C]elacridar with the unlabeled compound also increased the brain uptake in rats (210). Another PET study found increased K_1 values of [^{11}C]elacridar in P-gp and BCRP knockout mice compared to wild-type mice, suggesting the brain penetration of [^{11}C]elacridar is limited by these two transporters (211). In conclusion, [^{11}C]elacridar is not specific for P-gp and its radioactive signal in baseline scans is very low.

O-desmethyl-tariquidar was reacted with [^{11}C]methyl iodide or [^{11}C]methyl triflate to obtain [^{11}C]tariquidar (152,212). In vivo studies performed in mice showed an increase of [^{11}C]tariquidar brain uptake after the administration of unlabeled tariquidar (212). Moreover, the brain uptake of [^{11}C]tariquidar was higher in P-gp knockout mice and BCRP knockout mice than in wild-type mice (152,212). PET experiments performed in rats also found an increase in the brain uptake of [^{11}C]tariquidar after the administration of tariquidar and elacridar (152). The studies revealed a high metabolic stability of the tracer in the brain and plasma (212). In tumor cells, the uptake of [^{11}C]tariquidar and [^{11}C]elacridar increased after the administration of unlabeled tariquidar and this increase was similar to the one observed for [^{11}C]verapamil (213). The kinetics of [^{11}C]tariquidar and [^{11}C]elacridar were also evaluated in healthy human subjects. The brain uptake at baseline condition was very low for both tracers, however, after the administration of unlabeled tariquidar the brain uptake of [^{11}C]tariquidar increased by $27 \pm 15\%$ and by $21 \pm 15\%$ in the case of [^{11}C]elacridar. Both tracers showed a low amount of radiometabolites in plasma, 60 minutes after injection. The results indicate that [^{11}C]tariquidar and [^{11}C]elacridar behave as P-gp and BCRP substrates in vivo and therefore are unable to visualize P-gp expression at the BBB (214).

The radiosynthesis of [^{11}C]laniquidar involved methylation of a precursor with [^{11}C]methyl iodide in DMSO containing tetrabutylammonium hydroxide (151). [^{11}C]laniquidar was injected via the tail vein, and rats were sacrificed at different time-points to count radioactivity in various tissues. Moreover, rats were also pretreated with cyclosporine A and the brain concentration of [^{11}C]laniquidar was determined 30 minutes after injection. The brain uptake of [^{11}C]laniquidar was low, and was increased after treatment with cyclosporine A. The metabolism of the tracer was slow, resulting in a parent fraction of 68% in plasma, 30 minutes after injection. However,

the results suggest that laniquidar may act as a P-gp substrate instead of as a P-gp inhibitor at tracer concentrations (151). Co-administration of [^{11}C]laniquidar and unlabeled laniquidar in mice confirmed that laniquidar may act as a P-gp substrate at low doses and as a P-gp inhibitor at high doses (215).

[^{18}F]Fluoroelacridar [^{18}F]fluoroethyl-elacridar and [^{18}F]fluoroethyl-tariquidar

The synthesis of fluorine-18 labeled analogs of elacridar and tariquidar has been reported. These tracers showed similar properties to their carbon-11 analogs (216,217). [^{18}F]Fluoroelacridar was synthesized by nucleophilic aromatic substitution of the 1-nitro precursor. The reaction was done in the presence of dried kryptofix₂₂₂ and K_2CO_3 complex together with the precursor dissolved in dimethyl sulfoxide (DMSO) and the reaction mixture was heated for 60 minutes at 175 °C. Preclinical studies performed in rats showed a significant increase in brain uptake of the tracer after the administration of the P-gp and BCRP inhibitor elacridar. Brain uptake was also higher in P-gp and BCRP knockout than in wild-type mice (216). Although [^{18}F]Fluoroelacridar behaved similarly to [^{11}C]elacridar, both tracers are substrates of P-gp and BCRP which hampers the quantification of a specific transporter. [^{18}F]Fluoroelacridar also showed an intense defluorination in vivo, which limits its use as a PET tracer (216).

[^{18}F]fluoroethyl-elacridar and [^{18}F]fluoroethyl-tariquidar were synthesized by alkylation of its O-desmethyl precursors with [^{18}F]2-fluoroethyl bromide. These precursors were heated at 90-120°C for 10 minutes in DMF containing tetrabutylammonium hydroxide and [^{18}F]2-fluoroethyl bromide. Preclinical studies performed in wild-type and knockout mice revealed that these fluorine-18 tracers present similar kinetics and biodistribution as their carbon-11 analogs. An increase in the brain uptake was observed after the administration of tariquidar or elacridar to wild-type mice, and a higher uptake of the tracer was found in P-gp/BCRP knockout mice compared to wild-type (217). Both tracers were again substrates of P-gp and BCRP, therefore, they cannot be used to measure only the P-gp function (171,217).

The radiolabeling of P-gp inhibitors to measure the P-gp expression turned to be an unsuccessful strategy. The results provided by the preclinical and clinical studies were unexpected. First, the baseline brain uptake of these tracers was very low and, second, the treatment with P-gp inhibitors caused an increase in the brain uptake. At tracer concentrations, these compounds may act as P-gp substrates rather than P-gp inhibitors. (165). Therefore, [^{11}C]elacridar, [^{11}C]tariquidar and [^{11}C]laniquidar as well as their fluorine-18 analogs fail to measure P-gp expression at the BBB (208).

Novel P-gp inhibitors as PET tracers

Until now, most radiolabeled inhibitors aimed to quantify the expression of the P-gp transporter have not been successful. However, Verbeek and colleges have recently published the synthesis of two novel PET tracers for measuring P-gp expression, [^{18}F]2-(4-fluoro-2-oxoindolin-3-ylidene)-N-(4-methoxyphenyl)hydrazine-carbothioamide ([^{18}F]5) and [^{18}F]2-(6-fluoro-2-oxoindolin-3-ylidene)-N-(4-methoxyphenyl) hydrazine-carbothioamide ([^{18}F]6) (218). The kinetics of both tracers were evaluated in P-gp knockout mice and rats before and after administration of the P-gp inhibitor tariquidar. [^{18}F]5 showed greater metabolic stability than [^{18}F]6. PET studies performed in P-gp knockout mice revealed a significantly lower uptake of [^{18}F]5 compared to wild-type mice. Additionally, the administration of tariquidar did not modify the uptake of the tracer in the rat brain. Thus, this study suggested that [^{18}F]5 may be a promising radiotracer that binds to the P-gp transporter and may allow measurement of P-gp expression levels in vivo (218). However, additional experiments are needed to prove if [^{18}F]5 behaves as a P-gp inhibitor.

Kinetic Modeling of the P-gp Function

As has been explained before, PET is a functional imaging technique that measures the radioactivity concentration of a tracer in a specific target. This technique allows monitoring of the interactions of a radiotracer with its target(s) and quantification of the effects of a drug on physiological processes (219). Quantitative analysis of the PET data can involve the normalizing of average values from a region of interest using, for instance, standardized uptake values (SUV) or kinetic modeling methods. Kinetic modeling usually requires the acquisition of dynamic images, which give information about the concentration of the tracer in the target tissue as a function of time, as well as measurements of radioactivity in blood or plasma of the subject at different time points to acquire an arterial input function (220–222). Moreover, since radiotracers can be extensively metabolized, the radioactivity concentration in plasma should be corrected for the fraction of metabolites. Reference tissue models have also been developed in order to avoid the need for blood sampling. In this case, the time-activity curve of the radiotracer in the tissue of interest is related to the time-activity curve in a reference tissue that lacks the tracer target (222,223). Compartmental models are frequently used to describe the tracer kinetics, in order to analyze the delivery and elimination of the tracer in the tissue of interest. These methods can be used to calculate the mean transfer rate constants, which describe the movement of the tracer through different compartments, and the volume of distribution (V_T) or the binding potential of the tracer to the target (224). Kinetic modeling can also be used to determine which parameter

describes most adequately the function under study or which parameter is most sensitive to changes in this function (221).

The P-gp transporter is mainly located at the luminal side of the endothelial cells at the BBB. Thus, the rate constants K_1 and k_2 , which describe the movement of the tracer from the blood to the brain (or first tissue compartment), have been proposed as the best parameters to measure the P-gp function (156). Since the P-gp transporter is an efflux pump, it is expected that the efflux rate constant k_2 would be the parameter which is most affected by a P-gp inhibition, and thus, it would be the best parameter to measure the P-gp function. However, several PET studies with P-gp tracers found a significant increase in K_1 and V_T after administration of P-gp inhibitors, but no changes in k_2 . Thus, it was suggested that P-gp tracers pass from the blood to the endothelial cells and from there back to the blood by a very rapid P-gp-mediated transport. This transport is much more rapid than the PET acquisition, and therefore, the P-gp function is reflected in the value of K_1 , instead of the value of k_2 . A review of the kinetics of several P-gp PET tracers concluded that K_1 corrected for changes in the blood flow is the preferred parameter to measure P-gp function at the BBB (225). However, recent studies performed with [^{11}C]metoclopramide in non-human primates observed a significant decrease in k_2 after intravenous co-infusion of tariquidar. The results of these studies suggested that the most sensitive parameter to measure P-gp inhibition was V_T (206). Similar results were obtained in humans where a significant increase in V_T was found during the infusion of cyclosporine A. In that study, K_1 showed a significant increase in only a few brain regions after P-gp inhibition (207). These publications have re-opened the debate about the question which kinetic parameter represents most adequately the P-gp function.

The Use of P-gp Tracers and PET to Assess Brain Disorders in humans

The association between Alzheimer's disease and an impaired function of P-gp is an interesting field of research and was studied by various research groups. In the last decade, several radiotracers have been developed to evaluate the function of P-gp in Alzheimer's disease (226). Only a few of these tracers survived the in vitro and animal tests and were employed in humans. Until now, (*R*)-[^{11}C]verapamil is the gold standard to measure the function of P-gp in patient brains. This tracer revealed a significant decline of P-gp function in AD patients compared to healthy controls (132). However, (*R*)-[^{11}C]verapamil does not allow assessment of the efficacy of P-gp inducers which are considered as promising drugs for the treatment of AD. For this purpose, weak P-gp substrates are intensively studied and validated in rodents and non-human primates. The fact that these compounds have shown a higher uptake at baseline and a higher sensitivity to changes of P-gp function compared to avid substrates is encouraging (204,227,228).

In PD, a decreased P-gp function in frontal regions of the brain was seen only in advanced stages of the disease, using (*R*)-[¹¹C]verapamil. In the early stages of PD, no significant reduction of P-gp function was found, which makes P-gp dysfunction at the BBB less likely to be the primary cause of the disease (76,77).

In epilepsy, the function of P-gp is thought to be increased, which leads to insufficient uptake of antiepileptic drugs in the brain. Although [¹¹C]verapamil alone cannot accurately assess increases of P-gp in patients with epilepsy, [¹¹C]verapamil can be used in combination with tariquidar. This combination indicated that patients suffering from drug-resistant epilepsy show a higher P-gp function in some regions of the brain than drug-sensitive patients or healthy controls (78). Additionally, Langer and colleagues studied the P-gp function in a small group of patients suffering from drug-resistant epilepsy and they found some asymmetries in the kinetic parameters of [¹¹C]verapamil in the parahippocampal and ambient gyrus (PAHG), amygdala and medial and lateral anterior temporal lobe. However, these regional differences were not statistically significant (229). [¹¹C]Phenytoin, a weak substrate for P-gp, is under investigation as a hopefully better technique to evaluate the function of P-gp in patients suffering from drug-resistant epilepsy.

The Use of P-gp Tracers to Evaluate the Clinical Implications of Drug-Drug Interaction

ABC transporter-mediated drug-drug interactions (DDIs) have gained attention since numerous compounds can interact with these transporters.

On one hand, concomitant administration of drugs can culminate in adverse drug events which can cause patient hospitalization and increased patient morbidity as well as increased treatment costs. Interaction of a drug with ABC transporters can modify the pharmacokinetics of a co-administrated drug by changing its absorption, distribution, metabolism and/or elimination. DDIs can lead to unexpected toxicity or reduced drug effectiveness (230,231). For instance, a drug that inhibits the P-gp function can increase the concentration of a concurrently administered drug in the target tissue and can thus increase the risk of an adverse drug event. In the case of drugs with a narrow therapeutic index such as digoxin, which is a P-gp substrate, it is essential to study potential DDIs (231). However, it should be noted that not all DDIs will be clinically relevant (230).

On the other hand, transporter-mediated DDIs are crucial in the drug development process. The FDA and EMA have already advised *in vitro* assays to screen whether a new compound is a P-gp substrate, inhibitor or inducer (108,109). However, such *in vitro* methods have disadvantages.

They are not yet standardized among laboratories and P-gp inducers are difficult to recognize in vitro (230,231). Furthermore, for ABC transporters other than P-gp, in vitro methods have not yet been fully developed (231). In addition, if in vitro studies show that a new compound may interact with an ABC transporter, in vivo studies need to be performed to confirm this finding (164,231).

In clinical studies, the drug concentration in the blood of the subjects is usually measured. However, it is known that some DDIs mediated by ABC transporters do not result in a change of drug concentration in the blood, but changes in tissue pharmacokinetics. For example, P-gp inhibition at the BBB is usually not accompanied by changes in the plasma concentration of the drug but it causes an increase in brain concentration (164,232).

Since PET allows quantification of the concentration of the drug in the target tissue, imaging represents an interesting technique to study the DDIs mediated by ABC transporters. PET images can be used to quantify several pharmacokinetic parameters of a test drug, such as the tissue and blood concentrations during the scanning time. This usually requires the use of dynamic PET scans and blood sampling. By applying pharmacokinetic models to the data, different kinetic parameters are estimated which may help to interpret some physiological processes (164).

PET studies are commonly applied in drug development to measure the binding of a new drug to its target receptor (233–235). Moreover, as many drugs can be affected by the efflux action of ABC transporters and, therefore, do not reach the desired target, PET studies of ABC transporters may be used to predict which drug can reach the CNS in appropriate concentrations.

These PET studies require the administration of a radiotracer to image the function of a specific ABC transporter. Each study consists of a dual-scan protocol where the radiotracer is administrated under two different conditions. One scan will be performed without the administration of the test drug. It will provide information about the normal distribution of the tracer. This scan will be referred to as the baseline scan. The second scan will be performed after (or during) administration of the newly developed drug. Changes in the distribution of the radiotracer with regard to the baseline scan will show how the test drug affects the function of the specific ABC transporter. If the drug under study is expected to be a substrate of the ABC transporter, a different study protocol may be used. In this case, the drug under study could be radiolabeled with fluorine-18 or carbon-11. After studying the normal distribution of the test drug, a second PET scan should be performed in combination with a well-known inhibitor or inducer of the specific ABC transporter. This approach will show how changes in ABC transporter function affect the distribution of the test drug (164,232).

PET studies of ABC transporters play now an important role in the assessment of drug disposition in target tissues, in particular, the CNS. Therefore, the search for improved tracers for PET imaging of ABC transporters has been intensified (*138,155,165*).

Discussion and Concluding Remarks

The implication of ABC transporters in the onset of neurodegenerative disease as well as in the deposition of CNS drugs makes these efflux transporters an interesting therapeutic target. Since PET imaging allows the *in vivo* assessment of biochemical processes, this technique is well-suited for evaluation of the ABC transporter function under different conditions or at different stages of a disease. Therefore, many research groups are developing novel PET tracers for improved assessment of ABC transporter function *in vivo*. Current research focuses on the development of weak substrates for P-gp which can be used to measure both increases and decreases of the efflux function.

PET imaging of ABC transporters may expand our knowledge regarding their involvement in the onset and progression of several neurodegenerative diseases. Novel treatment approaches for Alzheimer's or Parkinson's disease, such as treatment with P-gp inducers, can also be tested in experimental animals and humans, using PET. Moreover, PET imaging may be a useful tool to evaluate the affinity of novel CNS drugs toward various ABC transporters. This technique may predict the biodistribution and, in particular, the brain penetration of new drug candidates at an early stage in the drug development process, and may thus enhance the success rate of drug development.

It is expected that the use of novel and specific tracers for ABC transporters will increase our understanding regarding the diagnosis and treatment of neurodegenerative disease as well as our insight into potential transporter-mediated DDIs.

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Chapter 3

Test-retest repeatability of [^{18}F]MC225-PET in rodents: A tracer for imaging of P-gp function

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Graphical abstract

Abstract

In longitudinal PET studies, animals are repeatedly anesthetized which may affect the repeatability of PET measurements. The aim of this study was to assess the effect of anesthesia on the P-gp function as well as the reproducibility of [^{18}F]MC225 PET scans. Thus, dynamic PET scans with blood sampling were made in 13 Wistar rats. Seven animals were exposed to isoflurane anesthesia one week before the PET scan (“Anesthesia-exposed” PET). A second group of six animals was used to evaluate the reproducibility of measurements of P-gp function at the blood–brain barrier (BBB) with [^{18}F]MC225. In this group, two PET scans were made with a 1-week interval (“Test” and “Retest” PET). Pharmacokinetic parameters were calculated using compartmental models and metabolite-corrected plasma as input function. “Anesthesia-exposed” animals showed a 28% decrease in whole-brain volume of distribution (V_T) ($p < 0.001$) compared to “Test”, where the animals were not previously anesthetized. The V_T at “Retest” also decreased (19%) compared to “Test” ($p < 0.001$). The k_2 values in whole-brain were significantly increased by 18% in “Anesthesia-exposed” ($p = 0.005$) and by 15% in “Retest” ($p = 0.008$) compared to Test. However, no significant differences were found in the influx rate constant K_1 , which is considered as the best parameter to measure the P-gp function. Moreover, Western Blot analysis did not find significant differences in the P-gp expression of animals no pre-exposed to anesthesia (“Test”) or pre-exposed animals (“Retest”). To conclude, anesthesia may affect the brain distribution of [^{18}F]MC225 but it does not affect the P-gp expression or function.

Keywords: Anesthesia, Blood-Brain Barrier, Isoflurane, P-glycoprotein, Positron Emission Tomography, Preclinical Studies, Test-Retest reproducibility.

1. Introduction

Delivery of many drugs to the central nervous system (CNS) is reduced by the action of efflux transporters in the blood-brain barrier (BBB), such as the breast cancer resistance protein (BCRP), and particularly P-glycoprotein (P-gp), i.e. ABCB1 (1). This transporter is known to limit the brain entry of antipsychotics, antidepressants, anti-epileptic drugs and chemotherapeutic agents (2). Several structurally diverse compounds act as substrates or modulators of P-gp. The transporter is, therefore, involved in drug-drug interactions (DDI) at the BBB with corresponding alterations of drug concentration, decreases in therapeutic efficacy and increases of toxicity (3,4). For this reason, the Food and Drug Administration (FDA) and European Medicines Agency (EMA) have developed specific guidelines for the screening of new drugs in order to determine their potential interactions with P-gp (5,6).

Since *in vitro* screening is based on simplified models of the living system, positron emission tomography (PET) imaging of the mammalian brain may provide important information concerning P-gp mediated drug transport and the mechanism underlying DDIs. Several PET tracers are available for this purpose, such as (*R*)-[^{11}C]Verapamil and [^{11}C]N-desmethyloperamide (7,8). Recently, [^{18}F]MC225 (5-(1-(2-[^{18}F]fluoroethoxy))-3-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-propyl)-5,6,7,8-tetrahydronaphthalene) was developed and validated for quantitative imaging of P-gp (9,10). This tracer is classified as a weak substrate of P-gp, resulting in higher brain uptake of radioactivity at baseline compared to other imaging agents (10). Inhibitors of P-gp were shown to increase the cerebral uptake of [^{18}F]MC225 (10).

Since PET imaging is minimally invasive, this technique seems well-suited for longitudinal studies, e.g. the scanning of a subject at baseline and after various interventions. However, PET imaging of small animals involves the use of anesthesia in order to immobilize the subject during the scan. Since it is well-known that anesthesia may affect the distribution of tracers in the CNS (3,11,12), it is important to examine the impact of anesthesia on the brain uptake of P-gp tracers. Moreover, the reproducibility of [^{18}F]MC225 PET scans should be assessed before longitudinal studies with this tracer can be performed.

The present study aimed to assess the influence of previous exposure to isoflurane anesthesia on P-gp function at the BBB and the reproducibility of [^{18}F]MC225 PET scans in rats. Pharmacokinetic parameters, such as the volume of distribution (V_T) and the rate constants (K_1 and k_2) of [^{18}F]MC225, were calculated using compartmental models and metabolite-corrected plasma radioactivity as input function. Western Blot (WB) analysis was performed to assess the expression of P-gp and BCRP transporters at the BBB.

2. Results and Discussion

PET imaging can provide insight into the physiology and pathophysiology of living brains (13). Moreover, it is a non-invasive technique that allows the monitoring of animals in longitudinal studies at several time points, before and after the onset of disease or treatment. Thus, each animal can serve as its own control, which reduces inter-subject variability (14). However, many factors may interfere with molecular mechanisms inside the brain and may have a negative impact on PET measurements. These include gender and strain differences of physiology and metabolism, the age of the animals, the composition of the diet or the duration of the fasting, the circadian rhythm and, the dose and kind of anesthetic (11). Therefore, the reproducibility of PET scans with a novel tracer should be evaluated before longitudinal studies are performed. Since anesthesia is commonly used to immobilize animals during acquisition of PET data, animals are repeatedly anesthetized in each longitudinal study design. Thus, it is necessary to examine the effect of anesthesia on the imaging target (in this case, cerebral P-gp function). To this aim, dynamic PET scans with [^{18}F]MC225 and blood sampling were made in 13 healthy male Wistar rats. In order to test the effect of the anesthesia on the P-gp function, seven animals were exposed to isoflurane anesthesia one week before the PET scan (“Anesthesia-exposed” PET). A second group of six animals was used to evaluate the reproducibility of measurements of P-gp function at the BBB with [^{18}F]MC225. In this group, two PET scans were made with a 1-week interval (“Test” and “Retest” PET).

2.1. Results

2.1.1. Tracer production

The tracer was obtained in an average synthesis time of 92 ± 7 min (mean $\pm$ SD) with a radiochemical yield of $4.9 \pm 0.9\%$ (decay-corrected). The radiochemical purity of [^{18}F]MC225 was higher than 99% and the molar activity higher than 29 TBq/mmol.

2.1.2. Plasma kinetics and metabolism of [^{18}F]MC225

Since the tracer was injected during a period of 1 min using a Harvard infusion pump, the concentration of [^{18}F]MC225 peaked at 62 seconds after the start of tracer injection in metabolite-corrected plasma time-activity curves (TACs).

Statistical analysis revealed that the plasma concentration of [^{18}F]MC225 (corrected for metabolites) was significantly higher in “Anesthesia-exposed” and “Retest” scans compared to “Test”. Figure 1A indicates a sharper rise of [^{18}F]MC225 in “Anesthesia-exposed” and “Retest” scans compared to “Test”. The parent fraction in plasma was also higher in “Anesthesia-exposed” and “Retest” scans than in “Test”, as can be observed in Figure 1B. Table 1 in the Supporting

Information shows the estimated marginal means (EMM) and standard errors (SE) calculated by Generalized Estimated Equation (GEE) analysis, and the statistical significance of differences between scans.

However, we did not observe any statistically significant differences in the biological half-life ($T_{1/2}$) of the tracer in the three scans (Anesthesia-exposed, Test, and Retest).

Figure 1: [^{18}F]MC225 concentration over the time course of the different PET scans: (A), metabolite-corrected time-activity curves in plasma (B) percentage of parent fraction as a function of time. Data are plotted as EMM $\pm$ SE.

2.1.3. Brain kinetics of [^{18}F]MC225

The SUV-TAC of the whole brain

Whole-brain TACs are shown in Figure 2. Although SUV values seem higher in the “Retest” group than in the other 2 groups, the statistical analysis did not indicate any significant differences between “Anesthesia-exposed” and “Test”, “Retest” and “Test”, or “Anesthesia-exposed” and “Retest”.

Figure 2: Whole-brain TACs of [^{18}F]MC225 at the three different scans.

Pharmacokinetic Modelling

Using all data of a 60-min scan, the 2TCM showed lower Akaike values than the 1TCM for all brain regions. Based on the AIC, the 2TCM should be the model of choice for data analysis; however, the standard errors (SE%) of the estimated rate constants K_1 and k_2 were lower in the 1TCM than in the 2TCM. The large SE% of K_1 and k_2 in 2TCM results in extremely high K_1 and k_2 values in all groups (more details in supplemental table 2). Due to the insufficient stability of a 2TCM fit, the 1TCM was selected as the preferred kinetic model for [^{18}F]MC225.

Statistical analysis of the data of 1TCM fits did not find significant differences in the K_1 values between the scans. However, the analysis found significant differences in the whole-brain volume of distribution (V_T) between the “Anesthesia-exposed” and “Test” scans (6.4 ± 0.4 vs 8.8 ± 0.3 ; $p < 0.001$) and also between “Retest” and “Test” (7.1 ± 0.4 vs 8.8 ± 0.3 ; $p < 0.001$) (Figure 3). Moreover, the analysis showed that the efflux constant k_2 was significantly higher in the “Anesthesia-exposed” scans than in “Test” (0.035 ± 0.002 vs 0.030 ± 0.001 ; $p = 0.005$), and in “Retest” scans than in “Test” (0.034 ± 0.001 vs 0.030 ± 0.001 ; $p = 0.008$). No significant differences in the V_T , k_2 or K_1 between the “Anesthesia-exposed” scan and “Retest” were found.

Figure 3: Parametric images of representative PET scans (Anesthesia-exposed, Test and Retest) showing voxel-wise values for K_1 of [^{18}F]MC225 calculated by 1-TCM

Figure 4: Parametric images of representative PET scans (Anesthesia-exposed, Test and Retest) showing voxel-wise values for V_T of [^{18}F]MC225 calculated by 1TCM.

A rise of k_2 may have caused a decrease of V_T in the “Anesthesia-exposed” and “Retest” scans (see figures 3 and 4).

Similar significant group differences in V_T and k_2 and a similar absence of significant differences in K_1 were observed in the studied brain regions. Figure 5 shows the $\text{EMM} \pm \text{SE}$ of K_1 , V_T and k_2 in the regions analyzed.

Figure 5: Regional K_1 (A) V_T (B) and k_2 (C) values of $[^{18}F]MC225$ at different scans. Data expressed as $EMM \pm SE$.

Several studies suggest that the best method to evaluate the P-gp function is to use the first part of the PET scan after the tracer injection (7,15). At early time points, the metabolism of the tracer is still limited and therefore radiometabolites may not interfere with the P-gp measurement. For this reason, the kinetic analysis was also performed using a 30-min scan duration. Similar results to the 60-min scan analysis were obtained. (see table 1).

Table 1: $EMM \pm SE$ V_T , K_1 , and k_2 of the selected brain region using 30-min scan duration and ITCM.

Region	Anesthesia-exposed PET			Test PET			Retest PET		
	Mean $K_1 \pm SE$	mean $k_2 \pm SE$	Mean $V_T \pm SE$	Mean $K_1 \pm SE$	mean $k_2 \pm SE$	Mean $V_T \pm SE$	Mean $K_1 \pm SE$	mean $k_2 \pm SE$	Mean $V_T \pm SE$
Amygdala	0.22 $\pm$ 0.01	0.047 $\pm$ 0	4.73 $\pm$ 0.24	0.25 $\pm$ 0.01	0.041 $\pm$ 0	6.13 $\pm$ 0.4	0.24 $\pm$ 0.02	0.045 $\pm$ 0	5.22 $\pm$ 0.24
Basal Ganglia	0.21 $\pm$ 0.02	0.05 $\pm$ 0	4.19 $\pm$ 0.22	0.24 $\pm$ 0.01	0.045 $\pm$ 0	5.55 $\pm$ 0.42	0.23 $\pm$ 0.02	0.053 $\pm$ 0	4.35 $\pm$ 0.22
Brainstem	0.33 $\pm$ 0.02	0.057 $\pm$ 0	5.87 $\pm$ 0.32	0.38 $\pm$ 0.02	0.05 $\pm$ 0	7.69 $\pm$ 0.39	0.36 $\pm$ 0.02	0.056 $\pm$ 0	6.49 $\pm$ 0.3
Cerebellum	0.3 $\pm$ 0.02	0.052 $\pm$ 0	5.74 $\pm$ 0.36	0.34 $\pm$ 0.01	0.047 $\pm$ 0	7.29 $\pm$ 0.32	0.32 $\pm$ 0.02	0.051 $\pm$ 0	6.33 $\pm$ 0.31
Corpus Callosum	0.2 $\pm$ 0.01	0.04 $\pm$ 0	5.06 $\pm$ 0.36	0.23 $\pm$ 0.01	0.035 $\pm$ 0	6.5 $\pm$ 0.33	0.22 $\pm$ 0.02	0.038 $\pm$ 0	5.66 $\pm$ 0.33
Cortex	0.2 $\pm$ 0.01	0.034 $\pm$ 0	5.98 $\pm$ 0.4	0.23 $\pm$ 0.01	0.029 $\pm$ 0	7.9 $\pm$ 0.42	0.22 $\pm$ 0.02	0.033 $\pm$ 0	6.8 $\pm$ 0.37
Hippocampus	0.21 $\pm$ 0.01	0.046 $\pm$ 0	4.53 $\pm$ 0.29	0.25 $\pm$ 0.01	0.041 $\pm$ 0	5.96 $\pm$ 0.31	0.23 $\pm$ 0.02	0.044 $\pm$ 0	5.19 $\pm$ 0.29
Hypothalamus	0.26 $\pm$ 0.02	0.048 $\pm$ 0	5.44 $\pm$ 0.29	0.3 $\pm$ 0.02	0.042 $\pm$ 0	7.15 $\pm$ 0.46	0.28 $\pm$ 0.02	0.048 $\pm$ 0	5.88 $\pm$ 0.26
Medial Geniculate	0.22 $\pm$ 0.02	0.054 $\pm$ 0	4.26 $\pm$ 0.37	0.26 $\pm$ 0.01	0.045 $\pm$ 0	5.92 $\pm$ 0.4	0.26 $\pm$ 0.02	0.053 $\pm$ 0	4.82 $\pm$ 0.23
Mesencephalic Region	0.28 $\pm$ 0.01	0.055 $\pm$ 0	5.2 $\pm$ 0.28	0.34 $\pm$ 0.02	0.051 $\pm$ 0	6.65 $\pm$ 0.39	0.31 $\pm$ 0.02	0.054 $\pm$ 0	5.88 $\pm$ 0.35
MidBrain	0.28 $\pm$ 0.02	0.055 $\pm$ 0	5.15 $\pm$ 0.3	0.33 $\pm$ 0.02	0.049 $\pm$ 0	6.77 $\pm$ 0.3	0.31 $\pm$ 0.02	0.053 $\pm$ 0	5.9 $\pm$ 0.35
Septum	0.22 $\pm$ 0.02	0.052 $\pm$ 0	4.23 $\pm$ 0.24	0.25 $\pm$ 0.01	0.045 $\pm$ 0	5.57 $\pm$ 0.43	0.23 $\pm$ 0.02	0.048 $\pm$ 0	4.92 $\pm$ 0.47
Striatum	0.2 $\pm$ 0.02	0.047 $\pm$ 0	4.35 $\pm$ 0.27	0.23 $\pm$ 0.01	0.038 $\pm$ 0	5.91 $\pm$ 0.39	0.22 $\pm$ 0.02	0.045 $\pm$ 0	4.77 $\pm$ 0.26
Superior Colliculus	0.28 $\pm$ 0.02	0.061 $\pm$ 0	4.69 $\pm$ 0.26	0.32 $\pm$ 0.02	0.054 $\pm$ 0	6.06 $\pm$ 0.41	0.31 $\pm$ 0.02	0.06 $\pm$ 0	5.21 $\pm$ 0.35
Thalamus	0.24 $\pm$ 0.02	0.058 $\pm$ 0	4.17 $\pm$ 0.22	0.28 $\pm$ 0.01	0.052 $\pm$ 0	5.37 $\pm$ 0.34	0.26 $\pm$ 0.02	0.057 $\pm$ 0	4.64 $\pm$ 0.29
Whole Brain	0.23 $\pm$ 0.02	0.044 $\pm$ 0	5.3 $\pm$ 0.31	0.26 $\pm$ 0.01	0.038 $\pm$ 0	6.89 $\pm$ 0.36	0.25 $\pm$ 0.02	0.043 $\pm$ 0	5.93 $\pm$ 0.31

2.1.4. Test- Retest analysis

The “Test” and “Retest” scans did not differ significantly regarding the injected dose (31 $\pm$ 5.7 MBq; $p=0.356$), the molar activity (58 $\pm$ 21.7 TBq; $p=0.868$) or the radiochemical purity (99.5 $\pm$ 0.4%; $p=0.350$). Animals in “Retest” scans were 1 week older than during the “Test”,

however, no significant differences in the body weight of animals between “Test” and “Retest” scans were found. (weight in “Test” scans=345±29 vs weight in “Retest” =366±31, $p=0.520$). The K_1 values were not significantly different between “Test” and “Retest” scans in any of the brain regions with the exception of the medial geniculate region and septum. Regarding the reproducibility evaluation, the Relative Difference (Rel. Diff. %) in K_1 between “Test” and “Retest” scans varied from -4 to -11% and the absolute variability or Test-Retest variability (TRV%) was lower than 12% in all the regions. The Coefficient of Variance (CV%) in “Test” scans showed an average for all the brain regions of 13±2% and in “Retest” scans of 20±3%. Even though the average of the Intraclass Correlation Coefficient (ICC) value for all the regions indicated a fair agreement between “Test” and “Retest” scans, most of the regions showed ICC values between 0.5-0.6, including the whole brain region, which indicated a moderate agreement. In the case of the V_T values, the t-test analysis found significant differences in all the brain regions between “Test” and “Retest” scans except for the septum. The Rel. Diff. % in V_T were larger than the ones from K_1 , ranging between 16% and 24% and TRV% was above 19% in all the brain regions. The between-subject repeatability expressed as CV% showed an average for all the regions of 10±4% in “Test” scans and 16±2% in “Retest” scan. Most of the ICC values for the V_T varied from 0.2-0.3 which indicated slight agreement between “Test” and “Retest” scans.

Table 2: Reliability of K_1 values between “Test” and “Retest” scans in all the brain regions.

Regions	K_1 Test± SD	K_1 Retest ± SD	Rel. Diff. K_1 (%)	TRV K_1 (%)	CV(%) K_1 test	CV(%) K_1 Retest	ICC K_1
Amygdala	0.24±0.04	0.21±0.04	-7.75	8.07	15.61	20.88	0.287
Cerebellum	0.34±0.03	0.3±0.04	-7.86	8.18	8.65	17.44	0.15
Corpus Callosum	0.23±0.04	0.19±0.03	-7.91	8.24	15.83	24.14	0.521
Medial Geniculate	0.26±0.04†	0.23±0.03†	-6.52	6.74	13.42	19.60	0.741
Mesencephalic Region	0.34±0.03	0.29±0.02	-9.95	10.47	9.48	14.89	-0.04
Septum	0.25±0.03†	0.2±0.03†	-11.07	11.72	12.72	26.20	0.537
Superior Colliculus	0.32±0.04	0.28±0.04	-5.90	6.08	12.26	18.83	0.493
Striatum	0.22±0.04	0.19±0.03	-7.89	8.22	16.70	23.79	0.616
Cortex	0.22±0.03	0.2±0.04	-4.01	4.09	13.44	23.11	0.692
Hippocampus	0.24±0.03	0.21±0.04	-8.86	9.28	14.34	22.80	0.555
Hypothalamus	0.3±0.04	0.26±0.05	-8.94	9.35	13.96	19.04	0.688
Mid Brain	0.33±0.05	0.28±0.03	-8.67	9.06	14.66	16.59	0.128
Brainstem	0.38±0.04	0.34±0.04	-7.98	8.31	10.01	14.78	-0.021
Thalamus	0.27±0.04	0.23±0.03	-7.53	7.82	15.02	20.94	0.428
Basal Ganglia	0.24±0.04	0.2±0.03	-9.18	9.62	15.40	21.83	0.37
Whole Brain	0.26±0.03	0.23±0.04	-6.65	6.88	12.38	20.42	0.522
Mean±SD			-7.92± 1.66	8.26± 1.79	13.37± 2.36	20.33± 3.32	0.42± 0.25

† Significant differences between Test and Retest scan ($p<0.05$)

Table 3: Reliability of the V_T values between “Test” and “Retest” scans in all the brain regions.

Regions	V_T Test $\pm$ SD	V_T Retest $\pm$ SD	Rel. Diff. V_T (%)	TRV V_T (%)	CV (%) V_T test	CV (%) V_T Retest	ICC V_T
Amygdala	8.22 $\pm$ 1.24*	6.28 $\pm$ 0.91*	-21.61	24.23	15.07	14.07	0.512
Cerebellum	9.17 $\pm$ 0.33*	7.42 $\pm$ 1.08*	-18.29	20.13	3.62	14.42	0.236
Corpus Callosum	8.31 $\pm$ 0.58*	6.49 $\pm$ 1.03*	-19.63	21.76	6.97	15.49	0.225
Medial Geniculate	7.01 $\pm$ 0.7*	5.49 $\pm$ 0.96*	-20.52	22.86	9.95	17.28	0.372
Mesencephalic Region	8.01 $\pm$ 0.82*	6.62 $\pm$ 1.11*	-16.13	17.55	10.19	16.58	0.533
Septum	7.04 $\pm$ 0.93	5.59 $\pm$ 1.29	-17.18	18.79	13.25	22.08	0.317
Superior Colliculus	7.44 $\pm$ 0.9*	6.05 $\pm$ 1.12*	-17.55	19.24	12.12	18.23	0.575
Striatum	7.35 $\pm$ 0.87*	5.55 $\pm$ 0.82*	-22.75	25.67	11.86	14.45	0.354
Cortex	10.31 $\pm$ 0.62 *	8.12 $\pm$ 1.25*	-20.09	22.34	5.97	15.23	0.259
Hippocampus	7.7 $\pm$ 0.62*	6.14 $\pm$ 0.93*	-18.16	19.97	8.07	14.76	0.338
Hypothalamus	8.99 $\pm$ 1.45*	6.92 $\pm$ 0.95*	-22.00	24.73	16.13	13.56	0.494
Mid Brain	8.19 $\pm$ 0.39*	6.77 $\pm$ 1.06*	-17.14	18.75	4.76	15.65	0.313
Brainstem	9.27 $\pm$ 1.04*	7.45 $\pm$ 1.09*	-19.50	21.61	11.26	14.61	0.57
Thalamus	6.66 $\pm$ 0.79*	5.22 $\pm$ 0.94*	-19.90	22.10	11.86	17.56	0.506
Basal Ganglia	6.85 $\pm$ 1.11*	5.06 $\pm$ 0.69*	-24.23	27.57	16.15	13.33	0.434
Whole Brain	8.81 $\pm$ 0.67*	7 $\pm$ 1.07*	-19.18	21.22	7.58	15.01	0.373
Mean $\pm$ SD			-19.62 $\pm$ 2.23	21.78 $\pm$ 2.75	10.3 $\pm$ 3.89	15.77 $\pm$ 2.2	0.4 $\pm$ 0.12

* Significant differences between Test and Retest scan ($p < 0.05$)

2.1.5. Blood flow analysis

The statistical analysis did not reveal any significant difference in whole-brain SUV values of the first frames among the three study scans (SUV “Anesthesia-exposed”=0.85 $\pm$ 0.049 vs SUV “Test”=0.84 $\pm$ 0.052; $p=0.876$ and SUV “Retest”=0.91 $\pm$ 0.025 vs SUV “Test”=0.84 $\pm$ 0.052; $p=0.276$). This result indicates that anesthesia does not cause long-term alterations of cerebral blood flow (CBF).

2.1.6. Western blot

Wistar rats with similar weights to the ones used during the PET scans were used for the WB analysis. These animals underwent the same procedures as the animals used in the PET studies: Group “Test” was once previously anesthetized, Group “Retest” was twice previously anesthetized and the group control was not subjected to anesthesia before the euthanasia. WB analysis showed no significant differences in P-gp and BCRP expression between the control group and any of the anesthetized groups (“Test” and “Retest” groups) (see figures 6 and 7).

Figure 6: Western Blot bands corresponding to P-gp, BCRP, and β -actin (160, 72 and 42 kDa predicted molecular weight respectively). Expression was relativized to β -actin and then normalized to protein expression found in control animals. Data are shown as mean $\pm$ SD (n per group = 4 with duplicates).

Figure 7: BCRP and P-gp expression in control animals, group “Test” (one time anesthetized) and group “Retest” (twice anesthetized).

2.2 Discussion

The plasma concentration of [^{18}F]MC225 (corrected for metabolites) was significantly higher in the scans where the animals were pre-exposed to anesthesia, “Anesthesia-exposed” (+25%) and “Retest” (+19%). Moreover, the parent fraction of plasma radioactivity was slightly but significantly increased by 6% in “Anesthesia-exposed” animals and by 5% in “Retest”, both compared to “Test”. However, we did not observe significant differences in $T_{1/2}$ among the scans. These results suggest that previous exposure to anesthesia may affect the tracer concentration in plasma but not its rate of elimination.

Previous PET studies in rodents have identified 1TCM as the preferred model to analyze [^{18}F]MC225 kinetics (10). In our own study, 1TCM was also chosen as the best model to fit the data. The model fit indicates a significant decrease in the V_T of [^{18}F]MC225 in whole-brain and

all analyzed brain regions of animals pre-exposed to isoflurane-anesthesia (“Anesthesia-exposed” and “Retest”). We also observed a significant increase in k_2 values of the tracer in whole-brain and in most brain regions analyzed. Whole-brain V_T was 28% lower in “Anesthesia-exposed” and 19% lower in “Retest” than in “Test” scans. Meanwhile, whole-brain k_2 was 18% and 15% higher in “Anesthesia-exposed” and “Retest” scans compared to “Test”. On the other hand, tracer levels in the blood were higher in animals pre-exposed to anesthesia (“Anesthesia-exposed” and “Retest” scans) than in “Test”. However, the whole-brain SUV-TACs in the 3 scans were not significantly different. These changes may be related, i.e. the decrease in V_T due to previous exposure to anesthesia may be caused by the increase in k_2 , resulting in reduced brain uptake and increased concentration of the radiotracer in plasma. However, the 1TCM fit did not indicate significant changes in the K_1 , which is considered by many authors as the best parameter to measure the P-gp function at the BBB (7,8,10,15–17).

Previous validation of [^{18}F]MC225 as a tracer for measuring P-gp function at the BBB of rats found a significant increase in the K_1 and V_T after the administration of the P-gp inhibitor tariquidar. This analysis also indicated that K_1 values were stable during the whole PET scan, whereas the V_T values became stable only after 30 min scan duration. Furthermore, after P-gp inhibition, the observed changes in K_1 were larger than the changes in V_T . Therefore, this study concluded that K_1 is the best parameter to measure the P-gp function, although V_T may also be used when sufficient scan data (> 30 min) are available (10).

In contrast to these findings, the pharmacokinetic evaluation of [^{11}C]Metoclopramide, another tracer considered as a weak P-gp substrate, indicated a significant decrease in the efflux constant k_2 values after the administration of tariquidar which caused a significant increase of the brain V_T in rats and non-human primates (18,19). The authors of these studies suggest the use of k_2 as the best parameter to measure the P-gp function. Nevertheless, it has been discussed that V_T and k_2 parameters can be affected by nonspecific trapping of the tracer and can also be affected by the fraction of radiometabolites inside the brain (7,15).

One strategy to avoid the interference of radiometabolites in the kinetic evaluation is to use short scan duration, such as 30-min scans. For instance, Muzi et al suggested the use of K_1 calculated with 1TCM and 30 min scan to evaluate the P-gp function at the human BBB using [^{11}C]verapamil (7). Following these suggestions, the kinetics of [^{18}F]MC225 were also evaluated using 30 min of scan data. The same results were obtained for 30-min and 60-min scans. The kinetic parameters such as V_T , K_1 , and k_2 were calculated using 1TCM which was selected as the preferred model. No significant changes in the K_1 were observed and the same significant decrease in the V_T and increase in k_2 were found in data of short scans.

All these results combined could suggest that isoflurane anesthesia induces or activates the P-gp function at the BBB. However, the absence of significant changes in K_1 refutes this hypothesis. Moreover, post-mortem analysis of brain tissue with WB did not indicate any significant change in P-gp or BCRP expression in animals pre-exposed to anesthesia. Thus, isoflurane anesthesia does not increase the expression of P-gp at the BBB.

Taken together, our results confirm that K_1 is indeed the best parameter to measure P-gp function at the BBB, since the lack of significant changes in K_1 in animals exposed to anesthesia corresponds to the absence of significant changes in P-gp expression shown by WB. Moreover, this study suggests that values of V_T and k_2 should be used with caution, since these values may change in longitudinal studies for unknown reasons.

The observed changes in V_T are in accordance with recent studies indicating that anesthesia may cause changes in the distribution or binding affinity of PET tracers (20). For example, the use of chloral hydrate and ketamine markedly increases, and pentobarbital decreases, the binding potential of [^{11}C]SCH23390 to dopamine D_1 receptors compared to that in conscious rats (12). Ketamine/xylazine also increases the binding potential of [^{11}C]MNPA to D_2 receptors compared to the binding in conscious animals (21). Chronic diazepam treatment reduces the global uptake of [^{18}F]FDG in the rat brain (22). Moreover, isoflurane anesthesia seems to affect the sensitivity of agonist tracers for dopamine D_2 receptors in an amphetamine challenge (23), and to alter the metabolism of the P-gp tracer [^{11}C]-N-desmethyl-loperamide (3). Furthermore, a prolonged effect of sevoflurane has been observed on the kinetics of [^{11}C]raclopride in non-human primates (24).

These changes can also be explained by changes in CBF (20). General anesthetics are known to affect CBF, e.g. propofol causing a decrease, whereas ketamine, isoflurane, sevoflurane and halothane cause a slight increase or do not affect global CBF (20,25–27). Since isoflurane is expected to increase CBF (28,29), the brain uptake of [^{18}F]MC225 should have increased in animals pre-exposed to anesthesia. However, we did not observe any increase but rather a significant decrease in V_T . Moreover, whole-brain SUV of the tracer in the initial frames and the K_1 values were not significantly different in the three study groups, suggesting that CBF was not significantly affected by previous exposure to anesthesia.

V_T values at “Test” and “Retest” were significantly different in most of the regions analyzed, whereas K_1 values were not. The K_1 values showed an overall TRV% lower than 10%; however, in the case of V_T , TRV% was around 20%. ICC values indicated a moderate agreement between Test- Retest K_1 values and only slight agreement for the V_T values. These results suggest that K_1 values show better reproducibility and reliability than V_T . Therefore, in longitudinal studies aimed to assess the P-gp function under different conditions, K_1 should be used.

The present study found a significant increase in k_2 values which led to a significant decrease of V_T values of [^{18}F]MC225 inside the brain of animals pre-exposed to anesthesia. However, the study did not find significant changes in the K_1 , which is considered as the best parameter to measure the P-gp function. These results were supported by WB experiment which did not find any significant increase in the P-gp or BCRP expression of animals pre-exposed to anesthesia. Overall, our results suggest that isoflurane-anesthesia affects the brain distribution of [^{18}F]MC225 causing changes in V_T and k_2 ; however, anesthesia does not alter P-gp expression at the BBB. Longitudinal studies with [^{18}F]MC225 are possible if K_1 is used to estimate the function of P-gp.

3. Methods

3.1. Chemicals

The precursor of [^{18}F]MC225, 5-[3-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-propyl]-5,6,7,8-tetrahydro-naphthalen-1-ol (MC226), was purchased from Syncom (Groningen, the Netherlands). Chemicals were purchased from Sigma-Aldrich (St.Louis, MO) and isoflurane from Pharmachemie (Haarlem, the Netherlands).

3.2. Tracer production

[^{18}F]MC225 was synthesized as previously described (9).

3.2. Animals

Thirteen healthy male Wistar rats were purchased from Janvier Labs (France). Before the start of the experiments, rats were acclimatized for at least seven days. Rats were housed individually after the first procedure. The room was kept at a constant temperature ($21 \pm 2^\circ\text{C}$) with a 12-12 h light-dark regimen. The rats were fed ad libitum and water was always available. Rat weight (354 ± 38 g) and behavior were monitored throughout the entire study. All applicable institutional and/or national guidelines for the care and use of animals were followed. The experiments were performed in compliance with Dutch and EU regulations. The protocol was approved by the National Committee on Animal Experiments of the Netherlands (CCD, the Hague) and the Institutional Animal Care and Use Committee of the University of Groningen (CCD license number: AVD105002015166, IvD protocol 15166-01-002).

Moreover, seventeen Wistar rats (Janvier Labs, France) were used for WB studies. Rats were acclimatized for at least seven days and were housed in groups (3 rats per cage). The rats were kept under controlled environment conditions (constant temperature of $22 \pm 1^\circ\text{C}$ and 12 h light / 12 h dark cycle and $60 \pm 5\%$ humidity). Food and water were allowed ad libitum. The weight

(354±26 g) and the behavior of the rats were monitored during the study. Experimental protocol was approved by the local Animal Care Committee according to the European Union (EU) rules (86/609/CEE, 2003/65/CE, and 2010/63/EU).

3.3. Study design

Regarding the PET studies, rats were divided into two groups. Group 1 was used to evaluate the effect of the anesthesia on the P-gp transporters and group 2 was used to assess the reproducibility of [^{18}F]MC225 data, with an interval of 1 week between the scans. On day 7, after arrival and acclimatization, group 1 animals (n=7) were transported to the PET facility and anesthetized with isoflurane in oxygen (5% for induction, 1.5–2% for maintenance, during 72±17 min), whereas group 2 animals (n=6) were transported but not subjected to anesthesia. On day 14, a dynamic PET scan with arterial blood sampling (60 min) was made for all rats. The scan of group 1 (made after previous exposure to anesthesia) will be referred to as “Anesthesia-exposed”, and the first scan of group 2 as “Test” (these animals had not been previously exposed to anesthesia). On day 21, a second dynamic PET scan was made for the rats of group 2. This scan is referred to as “Retest” (study design details in figure 8). All PET scans were performed under isoflurane anesthesia. Thus, “Retest” animals had been previously exposed to anesthesia, during their “Test” scan. Therefore, in “Anesthesia-exposed” and “Retest” PETs, the animals were anesthetized twice and in the “Test” only once. Before each scan, a cannula was placed in a side branch of the femoral artery for blood sampling during the scan (30). During this surgery, the rats were also under anesthesia for about 30 min. Anesthesia for surgery and PET scanning lasted 86–120 min. It is important to note that at the “Retest” scan, animals of group 2 had also been subjected twice to surgery (cannula placement), which may have induced extra stress. The body temperature of anesthetized animals was maintained close to the normal value with heating pads and electronic temperature controllers. Blood oxygenation and heart rate were continuously monitored during the scan, using pulse oximeters.

Figure 8: PET study design: group 1 (above) and group 2 (below).

Rats from WB studies were divided into three groups (figure 9). The “Test” group (n= 6) was exposed to isoflurane anesthesia only once, on day 14. The “Retest” group (n=6) was exposed twice to anesthesia, on days 7 and 14; and the control group (n=5) was not previously exposed to anesthesia. The anesthesia exposure consisted of 6 ± 1 min for induction (4%, 2.1 l/min) and 89 ± 5 minutes for maintenance (2%, 1.5 l/min). During maintenance, rats were anesthetized with a mask and the body temperature was controlled and kept close to normal values with a heating pad. On day 14, all rats were terminated by decapitation under deep anesthesia after phosphate buffered saline (PBS) perfusion and brains were immediately collected. The brain tissue was flash-frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ until samples were analyzed.

Figure 9: Western Blot study design.

3.4. PET Data collection

Anesthetized rats were placed in the PET camera (Focus 220; Siemens Medical Solution Inc.). The head of the rats was placed in the field of view.

First, a transmission scan was made using a ^{57}Co point source, for attenuation and scatter correction. Rats were then injected with [^{18}F]MC225 via a tail vein (32.5 ± 5.5 MBq, in 1 ml during 1 min, using a Harvard infusion pump) and the dynamic emission scan of 60 min was started simultaneously.

PET images were normalized and corrected for attenuation and decay. Emission sinograms were iteratively reconstructed using OSEM 2D, four iterations, and 16 subsets. The list-mode data of the emission scan was reconstructed into 21 frames (6×10 ; 4×30 ; 2×60 ; 1×120 ; 1×180 ; 4×300 ; 3×600 s).

3.5. Blood data collection

During the scan, blood samples (0.1–0.15 ml) were drawn from the arterial cannula at 10, 20, 30, 40, 50, and 60 s and 1, 1.5, 2, 3, 5, 7.5, 10, 15, 30 and 60 min after tracer injection. The total volume drawn was less than 2 ml. A 25 μl sample of whole-blood was used for radioactivity

measurements. The remaining volume of the samples was centrifuged at 6000 RPM (Mikro 20, Hettich, Germany) for 5 min to separate blood and plasma. Twenty-five μl of plasma were taken for radioactivity measurements and placed on ice to limit tracer metabolism. The radioactivity in blood and plasma was measured with a γ -counter (LKB Wallac, Turku, Finland) and values were corrected for decay.

The fractions of the parent [^{18}F]MC225 and of radioactive metabolites were determined using thin-layer chromatography (TLC) analysis. Plasma proteins were precipitated by the addition of acetonitrile (50 μl) to the plasma samples. After measurement of radioactivity in the γ -counter, plasma samples were mixed by vortexing for less than 1 min and were centrifuged for 5 min at 6000 RPM (Hettich Mikro 20, Hettich Zentrifugen, Germany) to obtain a protein-free supernatant. A 2.5 μl sample of each supernatant was spotted on a silica gel TLC plate. The TLC plate was eluted with 10% of MeOH in EtOAc and was placed on an activated phosphor storage plate overnight. The storage plates were read using a Cyclone® system (PerkinElmer Life and Analytical Science, Waltham, MA, USA). OptiQuant 03.00 software (PerkinElmer) was used to calculate the fractions of parent tracer ($R_f=0.8-0.9$) and radioactive metabolites ($R_f=0.2$).

3.6. Input function analysis

Measured radioactivity in blood samples was corrected for decay from the time of tracer administration. The time-activity curves (TAC) of whole-blood, plasma and metabolite-corrected plasma were expressed as standardized uptake value (SUV): $\text{SUV} = \text{radioactive concentration (MBq/ml)} / [\text{injected dose (MBq)} / \text{body weight (g)}]$. The metabolite-corrected plasma TAC-SUV was obtained by multiplying the SUV values of the plasma samples by the parent fraction.

A single exponential curve was fitted to the TAC-SUV of metabolite-corrected plasma (using values from 62 to 3564 seconds) by iterative non-linear least-squares approach using GraphPad software (GraphPad Prism version 7.02, California, USA) to calculate the biological half-life of the tracer ($T_{1/2}$): $Y = Y_0 \times \exp(-K_e \times X)$, where Y is the metabolite-corrected SUV value in plasma; Y_0 is the Y-intercept; K_e is the first-order rate constant of elimination and X the time. Tracer biological half-life was calculated as: $T_{1/2} = \ln(2)/K_e$ (31).

3.7. PET images processing

All PET images were automatically registered by rigid transformation to an [^{18}F]MC225 specific template, which was spatially aligned to a T2-weighted MRI of a Wistar rat in Paxinos space (32). Images were processed using PMOD v3.8 software (PMOD Technologies, Zürich, Switzerland). Sixteen volumes of interest (VOI) were selected from a rat brain atlas (32): amygdala, cerebellum, corpus callosum, medial geniculate, mesencephalic, septum, superior colliculus, striatum, cortex, hippocampus, hypothalamus, midbrain, brainstem, thalamus, basal ganglia, and the whole brain.

The radioactivity concentration was calculated for each selected brain region to generate TACs and it was expressed as SUV.

3.8. Pharmacokinetic Modeling

The TACs of whole-blood and plasma corrected for metabolites were used for pharmacokinetic modeling using PMOD v3.8. In this study 1TCM and 2TCM were evaluated using 30- and 60-min scan duration. The most appropriate model was selected based on the Aikake Information Criterion (AIC) which measures the goodness of the fit. The standard errors (SE%) of the estimated parameters were also taken into account during the model selection. The cerebral blood volume fraction was fixed to 5% following the recommendation from a previous preclinical study (10). The most appropriate model was used to calculate the influx (K_1) and efflux (k_2) rate constants and the volume of distribution (V_T).

3.9. Test-Retest analysis

The reproducibility of [^{18}F]MC225 scans was evaluated using the Rel. Diff. % between “Test” and “Retest” scans, TRV% between both measurements and the between-subject standard deviation was evaluated with the CV% for “Test” and “Retest” measurements. All these parameters were calculated according to methods of Elmenhorst et al. (33).

$$\text{Relative Differences (Rel. Diff \%)} = [(\text{Retest} - \text{Test}) / \text{Test}] * 100$$

$$\text{Test-Retest Variability (TRV\%)} = [(\text{Retest} - \text{Test}) / ((\text{Retest} + \text{Test})/2)] * 100$$

$$\text{Coefficient of Variance (CV\%)} = (\text{SD}/\text{mean}) * 100$$

The reliability of the measurements between and within subject was evaluated using the ICC. This analysis was performed using a 2-way mixed model with absolute agreement type and a confidence interval of 95%. IBM SPSS Statistics version 23 (Armonk, NY, USA) was used. ICC values between 0-0.2, 0.3-0.4, 0.5-0.6, 0.7-0.8 and 0.9-1 are categorized as slight, fair, moderate, substantial and almost perfect agreement (30,34).

3.10. Blood flow analysis

In the case of tracers with high extraction fraction, the early frames of a dynamic PET scan provide information about tracer delivery, and thus, about blood flow changes (35). Since [^{18}F]MC225 has a Log D of 3, it is considered as a lipophilic tracer with a high extraction fraction (9). Hence, in order to assess changes in blood flow after a previous exposure of animals to anesthesia, SUV values for whole-brain were calculated from frame 3 to 12 (from 20 to 300 seconds).

3.11. Western Blot Analysis

P-gp and BCRP expression were quantified by means of WB. Protein extraction was carried out adding cold RIPA buffer (Sigma, USA) with protease inhibitor cocktail (Sigma, USA) to brain slices in an approximated 10:1 proportion (volume/weight) and tissue disruption was performed in a TissueLyser II (Qiagen, Switzerland). Then tissue lysates were centrifuged for 30 minutes at 20000 g and supernatants were collected to perform WB. Protein concentration in the lysates was quantified using the Micro BCA Protein assay kit (ThermoFisher, USA).

The necessary volume of cell lysate containing a total amount of 50µg of protein was subjected to SDS-PAGE in 4-15% Criterion™ TGX™ Precast Midi Protein Gel (BioRad, USA) using a constant voltage of 140V. Then proteins were transferred onto a PVDF membrane (Millipore, Ireland) using a Trans-Blot semi-dry system (Bio-Rad, USA) with a limited voltage of 25V and 180 milliamps for two hours.

After the blotting step, membranes were blocked for 1 hour with a 3% Bovine Serum Albumin (BSA) solution (in Tris-Chloride Buffer with 0.1% of Tween 20 (TBST)). Once blocked, target proteins were detected through the incubation with primary antibodies against P-gp (Rabbit monoclonal to P Glycoprotein ab170904, Abcam, UK), BCRP (Rabbit polyclonal to BCRP/ABCG2 ab63907, Abcam, UK) and β -Actin (Mouse monoclonal to β -Actin ab8226, Abcam, UK) diluted 1:1000, 1:1000 and 1:5000 respectively in TBST with a 3% of BSA. Primary antibodies were incubated overnight at room temperature under agitation, and then membranes were washed three times in TBST in order to remove the excess of primary antibodies and avoid unspecific signaling. As secondary antibodies, an HRP-conjugated Goat anti-Rabbit IgG (P044801-2, Dako, Denmark) and HRP-conjugated Rabbit anti-Mouse IgG (P026002-2, Dako, Denmark) were used. They were diluted to 1:5000 in TBST with a 3% BSA and incubated for 1 hour at room temperature under agitation. Eventually, after washing, the HRP activity was revealed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher, USA) and detected in a Chemi Doc MP imaging system (BioRad, USA).

WB results were analyzed measuring the mean gray value of protein bands delimited in ROIs using ImageJ software (Rasband WS, USA). The relative expression of P-gp and BCRP to β -Actin was calculated for each sample, and the average of samples for each group was normalized to the control.

3.12. Statistical Analysis

Results are presented as mean $\pm$ SE or EMM $\pm$ SE unless otherwise indicated. IBM SPSS Statistics version 23 was used for statistical analysis. Group differences in V_T , K_1 , k_2 , SUV-TACs, and $T_{1/2}$ were assessed by generalized estimated equation (GEE) with the independent matrix (36). Results were considered statistically significant at $p < 0.05$, without correction for multiple comparisons.

The variability of V_T and the rate constant k_2 between “Test” and “Retest” (or “Test” and “Anesthesia-exposed”) was calculated using the “Test” scan as the reference, using the formulas: $(V_T(k_2) \text{ “Anesthesia-exposed”} - V_T(k_2) \text{ “Test”}) / V_T(k_2) \text{ “Test”}$ and $(V_T(k_2) \text{ “Retest”} - V_T(k_2) \text{ “Test”}) / V_T(k_2) \text{ “Test”}$. Both values are expressed as percentages. Differences in injected dose, molar activity, the radiochemical purity, and body weight between “Test” and “Retest” scans (Group 2) were evaluated using paired t -test and p values < 0.05 were considered statistically significant.

4. Supporting Information

The supporting information provides a table with values of tracer concentration in plasma, whole-blood and plasma-corrected for metabolites and the parent fraction in different groups. It also displays the statistical significance of differences between scans. A second table is also displayed in supporting information that provides Akaike values, $\text{SE}\%K_1$ and $\text{SE}\%k_2$ for 1TCM and 2TCM fits using 60-min scan duration.

Supplemental table 1: EMM $\pm$ SE values of whole-blood, plasma, metabolite-corrected plasma and percentage of parent fraction at different PET scans.

Scan	EMM $\pm$ SE	Comparison	p values
Whole-blood TAC			
Anesthesia-exposed	1.12 ± 0.04	Anesthesia-exposed-Test	0.018*
Test	0.96 ± 0.05	Test-Retest	0.001*
Retest	1.12 ± 0.03	Retest-Anesthesia-exposed	0.886
Plasma TAC			
Anesthesia-exposed	1.25 ± 0.05	Anesthesia-exposed-Test	0.009*
Test	1.02 ± 0.07	Test-Retest	0.002*
Retest	1.2 ± 0.07	Retest-Anesthesia-exposed	0.521
Metabolite-corrected plasma TAC			
Anesthesia-exposed	1.2 ± 0.05	Anesthesia-exposed-Test	0.003*
Test	0.96 ± 0.07	Test-Retest	$> 0.001^*$
Retest	1.14 ± 0.06	Retest-Anesthesia-exposed	0.482
% Parent Fraction throughout scan-time			
Anesthesia-exposed	80.39 ± 0.7	Anesthesia-exposed-Test	$> 0.001^*$
Test	75.53 ± 0.5	Test-Retest	0.005*
Retest	78.93 ± 1.07	Retest-Anesthesia-exposed	0.263
* Significant differences in the SUV-TAC between scans.			

Supplemental table 2: Mean $\pm$ SE of AIC, SE% V_T , SE% K_1 and SE% k_2 of all the brain region in the three different PET scan using 60-min acquisition time.

“Anesthesia-Exposed” PET								
1TCM					2TCM			
Regions	AIC $\pm$ SD	SE% V_T $\pm$ SD	SE% K_1 $\pm$ SD	SE% K_2 $\pm$ SD	AIC $\pm$ SD	SE% V_T $\pm$ SD	SE% K_1 $\pm$ SD	SE% K_2 $\pm$ SD
Amygdala	54.77 $\pm$ 6.33	9.75 $\pm$ 1.12	5.21 $\pm$ 0.86	12.55 $\pm$ 1.57	32.78 $\pm$ 8.53	9.23 $\pm$ 2.66	18.71 $\pm$ 20.65	49.06 $\pm$ 28.06
Cerebellum	51.92 $\pm$ 6.13	8.51 $\pm$ 1.14	4.73 $\pm$ 0.7	10.97 $\pm$ 1.46	9.33 $\pm$ 12.34	3.78 $\pm$ 0.78	8.07 $\pm$ 3.01	25.53 $\pm$ 7.45
Corpus Callosum	37.4 $\pm$ 7.24	6.71 $\pm$ 0.94	3.44 $\pm$ 0.6	8.6 $\pm$ 1.21	13.02 $\pm$ 13.52	5.02 $\pm$ 1.36	7.63 $\pm$ 2.45	39.84 $\pm$ 13.74
Medial Geniculate	80.5 $\pm$ 11.5	17.69 $\pm$ 4.73	9.92 $\pm$ 2.9	22.87 $\pm$ 6.3	75.27 $\pm$ 13.61	21.23 $\pm$ 11.05	76.93 $\pm$ 52.1	159.41 $\pm$ 75.91
Mesencephalic Region	50.08 $\pm$ 6.56	7.94 $\pm$ 1.39	4.49 $\pm$ 0.75	10.24 $\pm$ 1.75	26.52 $\pm$ 8.66	5.49 $\pm$ 1.25	12.96 $\pm$ 7.3	43.69 $\pm$ 16.31
Septum	63 $\pm$ 6.3	11.33 $\pm$ 1.82	6.24 $\pm$ 1.09	14.61 $\pm$ 2.4	54.28 $\pm$ 8.57	18.43 $\pm$ 12.7	14.39 $\pm$ 5.17	61.33 $\pm$ 21.39
Superior Colliculus	63.36 $\pm$ 5.32	10.88 $\pm$ 1.71	6.23 $\pm$ 0.91	14.06 $\pm$ 2.13	37.68 $\pm$ 9.49	6.89 $\pm$ 1.26	24.94 $\pm$ 18.62	54.29 $\pm$ 25.15
Striatum	43.06 $\pm$ 9.11	7.32 $\pm$ 1.54	3.95 $\pm$ 0.83	9.42 $\pm$ 1.94	19.84 $\pm$ 20.8	6.61 $\pm$ 4.68	7.32 $\pm$ 2.58	35.74 $\pm$ 15.43
Cortex	30.48 $\pm$ 5.98	6.15 $\pm$ 0.7	2.92 $\pm$ 0.45	7.82 $\pm$ 0.92	-5.64 $\pm$ 9.32	4.15 $\pm$ 1.45	5.02 $\pm$ 2.94	25.59 $\pm$ 5.36
Hippocampus	48.04 $\pm$ 7.82	8.3 $\pm$ 1.28	4.45 $\pm$ 0.92	10.69 $\pm$ 1.76	8.36 $\pm$ 13.22	4.67 $\pm$ 1.42	6.9 $\pm$ 3.42	24.84 $\pm$ 8.26
Hypothalamus	53.63 $\pm$ 9.43	9.13 $\pm$ 1.53	5.04 $\pm$ 1.18	11.79 $\pm$ 2.14	31.4 $\pm$ 8.06	7.3 $\pm$ 1.59	19.66 $\pm$ 16.3	52.24 $\pm$ 24.65
MidBrain	55.18 $\pm$ 5.25	8.97 $\pm$ 1.1	5.07 $\pm$ 0.6	11.58 $\pm$ 1.36	30.13 $\pm$ 11.89	6.49 $\pm$ 1.73	10.98 $\pm$ 4.94	40.97 $\pm$ 17.76
Brainstem	47.49 $\pm$ 2.62	7.23 $\pm$ 0.35	4.13 $\pm$ 0.32	9.35 $\pm$ 0.46	18.49 $\pm$ 21.16	5.32 $\pm$ 3.9	7.15 $\pm$ 2.66	32.41 $\pm$ 18.77
Thalamus	56.77 $\pm$ 8.31	9.48 $\pm$ 2.43	5.41 $\pm$ 1.4	12.25 $\pm$ 3.14	26.47 $\pm$ 8.26	6.01 $\pm$ 1.7	11.21 $\pm$ 7.07	33.28 $\pm$ 6.81
Basal Ganglia	47.32 $\pm$ 5.34	7.82 $\pm$ 1.27	4.31 $\pm$ 0.63	10.08 $\pm$ 1.58	28.84 $\pm$ 8.48	6.98 $\pm$ 1.53	13.71 $\pm$ 10.99	47.99 $\pm$ 20.63
Whole Brain	41.75 $\pm$ 4.86	7.1 $\pm$ 0.75	3.75 $\pm$ 0.5	9.12 $\pm$ 0.99	5.08 $\pm$ 14.09	3.21 $\pm$ 0.83	5.19 $\pm$ 2.94	20.54 $\pm$ 6.46

“Test” PET								
1-TCM					2-TCM			
Regions	AIC± SD	SE%V _T ±SD	SE%K ₁ ± SD	SE%K ₂ ± SD	AIC± SD	SE%V _T ± SD	SE%K ₁ ± SD	SE%K ₂ ± SD
Amygdala	60.23± 3.67	12.63± 3.67	5.93± 1.44	15.82± 4.43	36.09± 10.06	11.08± 4.67	15.57± 5.53	46.2± 9.77
Cerebellum	50.75± 1.49	8.73± 1.49	4.47± 0.63	11.03± 1.8	-0.32± 11.76	3.62± 1.83	5.98± 2.15	19.69± 4.2
Corpus Callosum	40.07± 1.23	7.81± 1.23	3.56± 0.51	9.77± 1.48	17.17± 8.1	7.9± 2.93	7.21± 1.81	37.16± 9.28
Medial Geniculate	68.95± 4.11	13.9± 4.11	7.18± 2.15	17.59± 5.22	64.02± 13.37	259709.42 ± 580689.72	44.79± 45.17	98.85± 56.68
Mesencephalic Region	57.33± 1.85	9.83± 1.85	5.21± 0.84	12.46± 2.25	28.07± 9.54	6.16± 1.86	11.76± 1.53	37.15± 7.58
Septum	71.02± 2.16	14.69± 2.16	7.41± 1.04	18.55± 2.68	61.74± 8.43	38± 54.88	64.25± 41.08	108.98± 55.66
Superior Colliculus	60.34± 3.55	10.94± 3.55	5.83± 1.84	13.86± 4.43	35.32± 9.63	7.15± 1.73	12.79± 8.15	45.39± 11.71
Striatum	44.38± 2.35	8.42± 2.35	4.02± 0.99	10.57± 2.87	19.87± 8.54	6.56± 2.71	22.86± 18.25	53.27± 24.01
Cortex	29.88± 1.9	7.11± 1.9	2.88± 0.68	8.78± 2.28	- 10.02± 9.84	3.75± 1.28	10.94± 10.96	33.01± 17.34
Hippocampus	49.2± 1.7	9.14± 1.7	4.41± 0.69	11.49± 2.1	17.68± 9.55	6.2± 2	8.77± 1.88	32.98± 10.65
Hypothalamus	52.53± 1.23	9.45± 1.23	4.69± 0.65	11.91± 1.54	31.35± 8.39	7.89± 1.94	18.81± 16.3	50.99± 27.04
MidBrain	55.55± 2.47	9.73± 2.47	5.07± 1.18	12.31± 3.05	28.99± 10.47	6.91± 1.9	11.92± 8.04	38.05± 8.45
Brainstem	49.85± 1.77	8.21± 1.77	4.32± 0.81	10.39± 2.17	10.05± 6.84	3.86± 0.87	6.74± 2.64	25.72± 3.78
Thalamus	52.66± 2.09	9.02± 2.09	4.74± 0.93	11.41± 2.55	20.59± 10.79	5.39± 1.43	7.65± 12.09	35.43± 15.71
Basal Ganglia	52.09± 0.9	9.23± 0.9	4.67± 0.42	11.66± 1.08	28.1± 4.31	6.84± 1.54	27.25± 23.58	56.69± 30.05
Whole Brain	42.01± 1.66	7.86± 1.66	3.72± 0.66	9.86± 2	-9.91± 10.73	3.11± 1.09	5.32± 2.22	19.51± 4.56

“Retest” PET								
1-TCM					2-TCM			
Regions	AIC± SD	SE%V _T ±SD	SE%K ₁ ± SD	SE%K ₂ ± SD	AIC± SD	SE%V _T ± SD	SE%K ₁ ± SD	SE%K ₂ ± SD
Amygdala	60.81± 1.76	11.5± 1.03	5.95± 0.27	14.95± 1.18	37.6± 4.69	8.36± 1.41	44.25± 30.64	79.07± 39.17
Cerebellum	53.75± 4.94	8.9± 0.83	4.89± 0.62	11.34± 1.12	8.07± 7.56	3.52± 0.66	10.45± 3.69	28.11± 8.89
Corpus Callosum	38.65± 4.46	7± 0.57	3.49± 0.36	8.85± 0.71	11.53± 12.59	4.4± 0.94	29.15± 34.15	71.65± 61.17
Medial Geniculate	75.83± 9.72	15.69± 4.59	8.61± 2.2	19.53± 5.66	66.05± 13.63	29.54±31 .51	35.44± 28.51	82.04± 39.21
Mesencephalic Region	54.39± 6.79	8.87± 1.27	4.97± 0.92	11.66± 1.73	27.79± 5.26	5.69± 1.44	26.96± 21.54	59.82± 31.15
Septum	63.22± 15.06	12.24± 3.89	6.58± 2.34	16.73± 5.02	51.66± 10.31	94098334 5.97± 23049290 16.4	13.38± 5	49.87± 10.36
Superior Colliculus	63.12± 9.29	10.99± 2.6	6.24± 1.53	12.97± 3.33	32.43± 10.62	6.51± 1.49	22.93± 25.17	44.33± 37.65
Striatum	48.22± 5.22	8.39± 1.02	4.42± 0.59	10.41± 1.3	19.05± 13.37	5.11± 2.14	30.52± 31.44	63.56± 43.69
Cortex	36.31± 6.94	7.28± 0.9	3.36± 0.52	9.02± 1.12	- 10.41± 13.13	3.18± 1.26	9.06± 8.01	29.38± 15.96
Hippocampus	48.46± 6.06	8.51± 1.15	4.43± 0.63	10.36± 1.45	11.89± 9.99	4.58± 1.03	12.83± 11.35	35.92± 20.17
Hypothalamus	59.39± 5.26	10.63± 1.61	5.68± 0.68	13.83± 1.94	33.07± 8.09	6.76± 1.61	37.2± 33.52	69.21± 44.16
MidBrain	58.33± 6.14	9.89± 1.63	5.49± 0.89	11.96± 2.04	31.28± 9.46	5.92± 1.4	31.3± 26.4	63.53± 32.77
Brainstem	53.87± 5.04	8.56± 0.91	4.83± 0.63	10.85± 1.23	15.88± 8.29	4.07± 1	15.49± 16.37	37.24± 21.89
Thalamus	53.88± 5.98	8.77± 1.32	4.94± 0.77	10.67± 1.67	30.06± 25.36	7.5± 7.02	14.02± 6.89	48.9± 25.92
Basal Ganglia	55.6± 5.3	9.52± 1.38	5.22± 0.68	11.69± 1.69	28.5± 10.47	6.18± 1.47	12.56± 8.17	35.8± 25.62
Whole Brain	45.9± 4.24	7.95± 0.55	4.12± 0.44	10± 0.74	-3.24± 9.25	3.04± 0.7	8.02± 3.82	24.67± 9.03

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Chapter 4

Pharmacokinetic modelling of [¹⁸F]MC225 for quantification of P-glycoprotein function at the blood-brain barrier in non-human primates with PET

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Abstract

[^{18}F]MC225 has been developed as a weak substrate of P-glycoprotein (P-gp) aimed to measure changes in the P-gp function at the blood-brain barrier (BBB) with positron emission tomography (PET). This study evaluates [^{18}F]MC225 kinetics in non-human primates and investigates the effect of both scan duration and P-gp inhibition. Three rhesus monkeys underwent two 91-min dynamic scans with blood sampling at baseline and after P-gp inhibition (8mg/kg tariquidar). Data were analyzed using 1-Tissue-Compartment Model (1-TCM) and 2-Tissue-Compartment Model (2-TCM) fits using metabolite-corrected plasma as input function, and for various scan durations (10-, 20-, 30-, 60- and 91-min). The preferred model was chosen according to the Akaike Information Criterion (AIC) and the standard errors (SE%) of the estimated parameters. For the 91-min scan duration, the influx constant K_1 increased by 40.7% and the volume of distribution (V_T) by 30.4% after P-gp inhibition, while the efflux constant k_2 did not change significantly. Similar changes were found for all evaluated scan durations. K_1 did not depend on scan duration (10-min- $K_1=0.2191$ vs 91-min- $K_1=0.2258$), while V_T and k_2 did. 10-min scan duration seems sufficient to properly evaluate the P-gp function using K_1 obtained with 1-TCM. For 91-min scan, V_T and K_1 can be estimated with a 2-TCM and both parameters can be used to assess P-gp function.

Keywords: Kinetic evaluation; PET; P-gp inhibition; P-gp tracer; rhesus macaque.

1. Introduction

The blood-brain barrier (BBB) is a physical membrane that separates the blood from the brain and is mainly composed of three cellular elements: endothelial cells, astrocytes end-feet, and pericytes (1,2). The cerebral endothelial cells connected by tight junctions impede the paracellular diffusion of hydrophilic compounds (1,3). Moreover, the BBB also expresses multiple influx and efflux transporters, which regulate the transport of exogenous and endogenous substances (4). Thereby, the BBB's main purpose is to protect the Central Nervous System (CNS) and ensure a controlled and stable environment for the correct neural function (4,5).

Members of the ATP-Binding Cassette (ABC) family are considered the most important efflux transporters at the BBB. Their main function is to protect the brain from harmful substances. P-glycoprotein (P-gp) is the best known ABC-transporter (4) and a wide variety of compounds have been identified as P-gp substrates or P-gp modulators. Therefore, P-gp is involved in various drug-drug interactions (DDIs) at the BBB. P-gp substrates are compounds that are transported by P-gp from the brain to the blood. P-gp modulators are compounds that can modify the P-gp function or expression. For instance, P-gp inhibitors increase the P-gp function by blocking the ligand-binding site of the P-gp transporters or by preventing the ATP hydrolysis. P-gp inducers increase P-gp expression by the activation of transcription factors such as the pregnane xenobiotic receptor (6–8). Increases of P-gp function can reduce the concentration of drugs inside the brain and therefore lead to decreased drug efficacy (8,9). On the other hand, decreases in P-gp function can cause increased concentrations of neurotoxic compounds inside the CNS and thus have been related to the onset of several neurodegenerative diseases (10–12).

Positron Emission Tomography (PET) with specific P-gp tracers is a suitable technique to measure the P-gp transporter function *in vivo* (13). Savolainen et al. (2017) introduced a fluorine-18 labeled tracer, called [^{18}F]MC225, which proved to be selectively transported by P-gp and not by BCRP, another important ABC transporter at the BBB (14). According to preclinical studies performed in mice and rats, this novel tracer behaves as a weak P-gp substrate. This results in higher brain uptake of [^{18}F]MC225 at baseline ($V_T=6.6-11$) compared with the widely used P-gp tracer (R)-[^{11}C]verapamil ($V_T=1.1-2.3$) (14–16). This elevated baseline uptake would allow measuring increases of P-gp function as may occur in patients with drug-resistant epilepsy (17) and also decreases of P-gp function in neurodegenerative diseases (18,19).

Based on a kinetic evaluation of [^{18}F]MC225 in rats, the 1-Tissue-Compartment Model (1-TCM) was selected as the model of choice for quantification of [^{18}F]MC225 transport across the BBB. This evaluation was performed using scans acquired at baseline and after tariquidar administration, which cause P-gp inhibition. The largest changes after P-gp-inhibition (compared to baseline) were found in the influx rate constant K_1 . For this reason, and in accordance with

previous publications, K_1 is considered as the most suitable parameter to estimate P-gp function at the BBB (15,20,21).

K_1 measures the transport of the tracer from the plasma to tissue. Based on the Fick principle and Renkin-Crone model (22,23), this parameter depends on blood flow and the unidirectional extraction fraction. Lipophilic tracers, such as [^{18}F]MC225 ($\log D=3.0$) (14), have a large extraction fraction and therefore K_1 is susceptible to changes in the blood flow (24). Moreover, errors in the input function, especially immediately after the injection of the tracer (plasma peak), can also affect the accuracy of K_1 estimation. Thus, more robust parameters, models and scanning procedures to assess the P-gp function should be investigated.

This study explores the pharmacokinetics of [^{18}F]MC225 in non-human primates (NHP), which approaches human conditions more precisely in terms of P-gp expression than rodents (25). To this aim, [^{18}F]MC225 kinetics were assessed at baseline and after P-gp inhibition, using dynamic PET scans with arterial blood sampling. We assessed the effect of varying scan durations and P-gp inhibition on kinetic model performance. Moreover, we compared the suitability of K_1 and V_T to measure P-gp function in terms of capturing the effects of P-gp inhibition and parameter robustness at various evaluated scan durations.

2. Experimental Section

2.1. Tracer production

The precursor 5-[3-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-propyl]-5,6,7,8-tetrahydronaphthalen-1-ol (MC226) and the cold reference compound, 5-(1-(2-fluoroethoxy))-3-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-propyl]-5,6,7,8-tetrahydronaphthalen (MC225), were supplied by the University of Bari, Italy. Tariquidar methanesulfonate hydrate was purchased from MedChemExpress (Monmouth Junction, NJ, USA). [^{18}F]MC225 was synthesized on a multipurpose synthesizer (F120, Sumitomo Heavy Industries, Tokyo, Japan) as previously described (14,15).

2.2. Animals

The study was performed at the Central Research Laboratory, Hamamatsu Photonics (Hamamatsu, Japan) in collaboration with the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). Three healthy-male rhesus monkeys (*Macaca mulatta*; Hamri, Ibaraki, Japan) were used. Monkeys were individually housed in U.S. National Institute of Health (NIH) standard adapted stainless steel cages, in a controlled room with a temperature of 24 ± 4 °C, a humidity 50

$\pm 20\%$, and under a 14-hour light/10-hour dark cycle. They were fed with 120 g of chow (Certified Primate Diet 5048, PMI Nutrition) in the morning and 100 g of raw sweet potato in the evening. The weight (7.26 ± 0.78 Kg) and the behavior of the animals were monitored during the study. The study was carried out in accordance with the recommendations of the NIH, the guidelines of the Ethics Committee of the Central Research Laboratory, Hamamatsu Photonics (approval HPK-2016-07A) and the Institutional Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology (approval 16067).

2.3. Data collection

2.3.1. Experimental design

All animals underwent two 91-min dynamic PET scans using [^{18}F]MC225: at baseline and after P-gp inhibition, within one month. P-gp inhibition consisted of intravenous (i.v.) injection of the P-gp inhibitor tariquidar (26–28) (8 mg/kg of body weight) 15-min before the PET scan. Eleven mg of tariquidar methanesulfonate hydrate was dissolved in 3 ml of saline as a suspension solution. The injection volume was adjusted to the weight of each monkey. The tariquidar methanesulfonate hydrate solution was slowly infused through a catheter inserted into the saphenous vein.

2.3.2. PET/MRI Data

A structural T1 weighted MRI scan (Signa Excite HDTx 3.0T, GE Healthcare, Milwaukee, WI, USA) was made before the first PET scan. All animals underwent 91-min dynamic PET scan (SHR-38000, Hamamatsu Photonics, Shizuoka, Japan) with arterial blood sampling. Animals were anesthetized (2.5% sevoflurane) during their transport but remained awake during the scans with their head immobilized using a fixation device. The monkeys were positioned in the camera in a sitting position, with stereotactic coordinates aligned parallel to the orbitomeatal plane.

A transmission scan was performed before the acquisition of the PET data using a rotating $^{68}\text{Ge}/^{68}\text{Ga}$ rod source (60-min), and its information was used for attenuation and scatter correction of the PET images. Animals were injected with [^{18}F]MC225 (684 ± 64 MBq) at the start of the emission scan, via the saphenous vein over a period of 30 seconds as a single bolus.

PET images were reconstructed using Filtered Back Projection with a Hanning filter of 4.5 mm and were composed of 49 frames (6×10 , 6×30 , 12×60 , and 25×180 seconds).

2.3.3. Blood Data

After the administration of the tracer, 19 blood samples (0.5 ml) were drawn from a cannula placed in the posterior tibial artery (at 8, 16, 24, 32, 40, 48, 56, 64 seconds and 1.5, 2.5, 4, 6, 10,

20, 30, 45, 60, 75, 91 minutes (min)). Then, plasma and blood were separated by centrifugation (12,000 rpm, 60 s) and the radioactivity was measured using a gamma-counter (1480 Wizard, PerkinElmer, Waltham, MA, USA).

Parent fraction and radioactive metabolites of [^{18}F]MC225 were analyzed following tracer injection (16, 40, 64 seconds and 6, 10, 30, 45, 60, 75, 91 min). Ethanol was added to the plasma fraction to precipitate the plasma proteins. The supernatant of these samples was analyzed using thin-layer chromatography plates (silica gel 60 F254, Merk, Millipore, Burlington, MA, USA) with a mobile phase of methanol/ethyl acetate (1/9). The parent (Retention factor (R_f) = 0.4) and metabolized fraction (R_f = 0) were assessed using a phosphor imaging plate and a bioimaging analyzer (FLA-7000, Fuji Film, Tokyo, Japan).

The percentage of the metabolites in plasma was calculated for each subject by fitting a single exponential equation to the values obtained from the metabolite analysis, using an iterative non-linear least squares approach using GraphPad software (GraphPad Prism version 7.02, CA, USA): $Y = (Y_0 - \text{plateau}) * \exp(-K_e * X) + \text{plateau}$ (29), where Y is the percentage of parent fraction at different time points, Y_0 is the intercept which was fixed to 100% (percentage of parent fraction at the beginning of the scan), K_e is the first order elimination constant, and X the time.

2.4. Data analysis

2.4.1. Input function

The radioactivity measured in blood samples was corrected for decay from the time of tracer administration, and time-activity curves (TAC) of whole-blood and plasma were calculated using standardized uptake values: $\text{SUV} = \text{Radioactive Concentration at time (T) (KBq/mL)} / (\text{injected dose (MBq)} / \text{body weight (Kg)})$. The metabolite-corrected plasma TAC was calculated using SUV from plasma samples multiplied by the percentage of parent fraction, as described above.

A semi-logarithmic plot of the tracer concentration in plasma, corrected for metabolites, versus time was performed to assess the compartmental model for plasma kinetics. The tracer elimination from the plasma was described with a 1-compartment model. Therefore, the rate constant of elimination (K_e) and the biological half-life ($T_{1/2}$) were calculated by fitting a single exponential curve, $Y = Y_0 * \exp(-K_e * X)$, to the metabolite-corrected plasma TAC of each subject, by an iterative non-linear least squares approach using GraphPad software, where Y is the SUV value in plasma-corrected for metabolites; Y_0 is the intercept; K_e is the first order elimination constant and X the time. Y_0 was fixed to the concentration values at 32 seconds after tracer injection (plasma peak). The half-life of the tracer was calculated as $T_{1/2} = \text{Ln}(2)/K_e$ (30).

2.4.2. PET/MRI

Images were processed using PMOD v3.8 software (PMOD Technologies, Zürich, Switzerland). All scans were registered to a reference MRI template defined in the Montreal Neurological Institute (MNI) monkey space (31–33). The individual MRI was registered to the reference MRI using a 3 probability map normalization (33,34). Then, [^{18}F]MC225 PET images were aligned to their corresponding MRI by rigid transformation, using a summation of all frames (supplementary material). Head motion correction was not applied to the data.

Several volumes-of-interest (VOIs) were selected from a brain atlas (32): basal ganglia, brainstem, cerebellum, cingulate cortex, hippocampus, hypothalamus, insular cortex, midbrain, occipital cortex, orbito-frontal cortex, parietal cortex, striatum, temporal cortex, thalamus and white matter. Additionally, a VOI covering the whole brain was included.

2.4.3. Pharmacokinetic analysis

Metabolite-corrected plasma and whole-blood TACs were used as input function to perform pharmacokinetic modelling using PMOD v3.8 software.

During pharmacokinetic analyses, blood delay was first corrected by estimating the delay of the whole brain TAC and then fixing that value for the rest of the regions. Different plasma input compartment model fits were evaluated, including 1-Tissue Compartment Model (1-TCM), 2-Tissues Compartment Model (2-TCM), and irreversible 2-TCM ($k_4=0$) with scan durations of 10-, 20-, 30-, 60-, and 91-min. The effect of fractional volume of blood (vB) for each model was explored by either fixing the value to 3, 4, 5, 6, and 7% or used as a fit parameter. The selection of the most appropriate model for each scan duration was based on the Akaike Information Criterion (AIC) and the standard errors (SE%) of the estimated parameters. Finally, changes in the pharmacokinetic parameters of each kinetic model between baseline and after-inhibition were also explored for all scan durations.

During the pharmacokinetic modelling, no constraints were applied to the fit parameters. However, those parameters with SE% higher than 1000% were considered unreliable and excluded for further analysis.

2.4.4. Parametric Images

Parametric images were calculated using a basis function implementation of the 2-TCM for illustrative purposes only (figure 5). The metabolite-corrected plasma TAC was used as the input function for 2-TCM (vB=6%), using PMOD. Parametric images were made for one subject using 91-min scan durations and the baseline and after P-gp inhibition scans.

2.5. Statistical Analysis

Descriptive data are presented as mean $\pm$ standard deviation (SD) and results of the statistical analysis are shown as Estimated Marginal Mean (EMM) $\pm$ standard error (SE). Statistical analysis was performed using IBM SPSS Statistics version 23 (IBM, Armonk, NY, USA). For each region, differences in AIC, SE%, pharmacokinetic parameters (V_T , K_1 , k_2), and blood kinetics (K_e and $T_{1/2}$) between scans and among different scan duration were assessed by Generalized Estimated Equation (GEE) with independent matrix (35). Within the GEE, different values for fixed vB, scan conditions, different kinetic models, and scan durations were selected as independent variables in the model. Results were considered statistically significant at $p < 0.05$, without correction for multiple comparisons. Differences in observed kinetic parameters because of P-gp inhibition were expressed as percentage change relative to the baseline values.

3. Results

3.1 Tracer production

[^{18}F]MC225 was produced with a radiochemical purity of $97.6 \pm 1.0\%$ and a molar activity higher than 36 GBq/ μmol . The characteristics of the tracers are summarized in supplemental table 1.

3.2 Input function

At 30 min after tracer injection, the parent fraction of plasma radioactivity was 53% in baseline and 48% in after-inhibition scans. Figure 1 shows the TAC of metabolite-corrected plasma and the percentage of [^{18}F]MC225 parent tracer at baseline and after-inhibition. The statistical analysis did not find significant differences regarding the biological $T_{1/2}$ and K_e of [^{18}F]MC225 between baseline and after-inhibition scans (table 1).

Figure 1: Mean $\pm$ SD Metabolite-corrected plasma TAC (A) and percentage of parent [^{18}F]MC225 (B) at baseline and after-inhibition scans.

Table 1: Tracer kinetics calculated from the metabolite-corrected plasma TAC.

Subject	Scan	K_e (seconds $^{-1}$)	$T_{1/2}$ (seconds)
Subject 1	Baseline	0.015	45.73
Subject 1	After-inhibition	0.013	52.84
Subject 2	Baseline	0.015	45.68
Subject 2	After-inhibition	0.014	49.68
Subject 3	Baseline	0.016	42.08
Subject 3	After-inhibition	0.017	40.51

K_e = elimination constant, $T_{1/2}$ = biological half-life.

3.3 Plasma input models: Compartmental models

3.3.1. Blood Volume Estimation:

The estimation of v_B during kinetic analysis provided values that ranged from 6 to 8%. No significant differences were found between v_B at baseline and after-inhibition (v_B baseline = 0.069 ± 0.002 and v_B after-inhibition = 0.057 ± 0.015); $p = 0.373$). When assessing the goodness of the fit, the lowest Akaike values were found with a fixed v_B of 6% (whole-brain AIC = -46 ± 3.9) and the free v_B (whole-brain AIC = -41 ± 4.9), without a statistically significant difference between both. Similarly, the lowest SE% for K_1 was found using either a fixed v_B of 6% or v_B as a free fit parameter. K_1 values were similar for all v_B s tested. In fact, the differences in estimated K_1 when using a fixed v_B (3-7%) or free v_B were smaller than 3%. Since fixing v_B did

not substantially improve the fit quality and had a minor impact on the pharmacokinetic parameters, it was decided to use v_B as a free fit parameter for the rest of the study (more information in table 2).

Table 2: $EMM \pm SE$ of AIC, $SE\%K_1$, and K_1 of the whole-brain region using different v_B .

Whole-Brain region			
v_B	AIC	$SE\%K_1$	K_1
3%	-6.4 ± 2.27	2.33 ± 0.04	0.23 ± 0.01
4%	-24.57 ± 3.42	1.77 ± 0.08	0.23 ± 0.01
5%	-32 ± 7.12	1.55 ± 0.2	0.22 ± 0.01
6%	-45.87 ± 3.95	1.22 ± 0.07	0.22 ± 0.01
7%	-34.66 ± 6.46	1.61 ± 0.22	0.22
Free	-40.92 ± 4.87	1.47 ± 0.14	0.22 ± 0.01

3.3.2. Model selection for different scan durations

In all evaluated scan durations, 2-TCM showed significantly lower AIC values than 1-TCM (supplemental table 2). Differences in AIC obtained with 1-TCM and 2-TCM increased with longer scan durations. Overall, for the 10-min scans, the AIC with 1-TCM was 49% higher than the one with 2-TCM and for the 91-min data, the AIC with 1-TCM was 146% higher than that obtained with 2-TCM. For all scan durations, the 1-TCM showed significantly lower $SE\%K_1$ than the 2-TCM (70% lower in 10-min scan and 6% lower in 91-min). The $SE\%V_T$ was extremely high in 2-TCM for short scan durations, providing unrealistically high V_T (Supplemental table 3).

Estimated parameters with $SE\%$ higher than 1000% were excluded from the analysis. This occurred in 22 out of 1440 occasions and was seen for V_T and k_4 based on the 2-TCM for short scan (10- to 30-min) durations and in regions such as hippocampus, brainstem, and striatum. The analysis also showed that the difference in $SE\%V_T$ between 1-TCM and 2-TCM decreased with longer scan duration, ranging from 92% difference in 10-min to 32% in 91-min scans.

While 2-TCM showed the lowest AIC values, due to the high $SE\%$ found in the estimation of V_T and k_4 , the 2-TCM was discarded as a robust model for short scans (10- to 30-min) but remained the model of choice for 60- and 91-min scan durations. Figure 2 shows representative 1-TCM and 2-TCM fits of the whole brain region.

Figure 2: Representatives 1-TCM (dashed line) and 2-TCM (solid line) fits of the whole brain at baseline (blue) and after-inhibition (red) scan, the black circles and squares represent the baseline and after-inhibition TACs respectively.

3.3.3. Effect of scan duration on the pharmacokinetic parameters

Figure 3 shows K_1 , V_T , and k_2 of the whole-brain region for different scan durations at baseline and after-inhibition. Based on the results above, the estimation of K_1 and V_T for each tested scan duration was performed with 1-TCM for short (<30-min) and 2-TCM for long scans (>60-min). K_1 remained relatively unaffected by varying the scan duration, while for V_T , the differences among the scan durations were more pronounced with a 62% lower V_T at 10-min than at 91-min. The k_2 values varied randomly across the various tested scan durations.

Figure 3: Boxplot showing the third quartile and first quartile range of V_T (A), K_I (B) and k_2 (C) values of the whole-brain region at baseline and after-inhibition scans in different scan durations. The black line within the box marks the median and the whisker.

3.3.4. Effect of P-gp inhibition

Figure 4 displays the mean values of V_T , K_I , and k_2 of all regions for the three subjects at baseline and after-inhibition, using the 91-min scan duration. As can be observed, V_T and K_I increased in all brain regions after tariquidar administration, displaying a similar pattern per brain region in baseline and after-inhibition scans. The K_I was the parameter most affected by P-gp inhibition. In 91-min scan duration, the whole-brain region K_I increased by 40.7% from 0.19 ± 0.01 at baseline to 0.26 ± 0.01 in after-inhibition scans, whereas the V_T increased from 10.6 ± 0.5 to 13.8 ± 0.1 , an increase of 30.4%.

Figure 4: Mean $\pm$ SE of V_T (A), K_1 (mL/mL/min) (B) and k_2 (1/min) (C) for all the regions at baseline and after-inhibition scans using 91-min scan duration.

In 2 of the 3 subjects, the k_2 values remained similar in baseline and in after-inhibition scans, whereas one subject showed an increase in the k_2 values after the P-gp inhibition. However, the overall k_2 values did not significantly change after the treatment with tariquidar in any brain region. Figure 5 shows illustrative V_T , K_1 , and k_2 parametric images at baseline and after-inhibition using 91-min scan.

V_T and K_1 increased significantly in all regions for all scan durations and subjects after the P-gp inhibition (see supplemental tables 4, 5, 6, and 7), while k_2 did not. Table 3 shows the K_1 and V_T values at baseline and after-inhibition as well as the relative change (%) due to P-gp inhibition in 30-min scan duration.

Figure 5: Parametric images calculated using 91-min scan duration and 2-TCM at baseline (A) and after-inhibition (B).

Table 3: EMM $\pm$ SE of V_T and K_1 at baseline and after-inhibition in 30-min scan duration for all the regions and p values of the difference between baseline and after-inhibition scans.

Scan duration 30-min				
Region	V_T baseline $\pm$ SE	V_T after-inhibition $\pm$ SE	% change V_T	p value
Basal Ganglia	5.5 $\pm$ 0.4	7.9 $\pm$ 0.2	45	p<0.001
Brainstem	5.5 $\pm$ 0.7	6.4 $\pm$ 0.3	15.9	p=0.321
Cerebellum	7.5 $\pm$ 0.9	8.3 $\pm$ 0.3	11	p=0.419
Cingulate Cortex	6.4 $\pm$ 0.5	8.7 $\pm$ 0.2	36.5	p<0.001
Orbito-Frontal Cortex	5.8 $\pm$ 0.3	8 $\pm$ 0.3	36	p=0.001
Hippocampus	6 $\pm$ 0.6	7.5 $\pm$ 0.3	24.1	p<0.001
Hypothalamus	5.7 $\pm$ 0.4	7.4 $\pm$ 0.3	29.5	p=0.013
Insular Cortex	6 $\pm$ 0.1	9.2 $\pm$ 0.4	54.1	p<0.001
Midbrain	5.9 $\pm$ 0.4	8.7 $\pm$ 0.2	46.3	p<0.001
Occipital Cortex	6.4 $\pm$ 0.4	8.4 $\pm$ 0.2	30.4	p<0.001
Parietal Cortex	6.1 $\pm$ 0.3	8.5 $\pm$ 0.3	39.5	p<0.001
Striatum	5.5 $\pm$ 0.4	8.2 $\pm$ 0.2	48.1	p<0.001
Temporal Cortex	6.2 $\pm$ 0.5	8.2 $\pm$ 0.3	31.8	p=0.003
Thalamus	5.7 $\pm$ 0.4	8.2 $\pm$ 0.3	45.9	p<0.001
White Matter	5.6 $\pm$ 0.4	7.7 $\pm$ 0.2	37.3	p<0.001
Whole Brain	6 $\pm$ 0.4	8 $\pm$ 0.2	33	p=0.001
Region	K_1 baseline $\pm$ SE	K_1 after-inhibition $\pm$ SE	% change K_1	p value
Basal Ganglia	0.15 $\pm$ 0.01	0.22 $\pm$ 0.01	44.9	p<0.001
Brainstem	0.14 $\pm$ 0.01	0.21 $\pm$ 0.01	49	p<0.001
Cerebellum	0.19 $\pm$ 0.01	0.27 $\pm$ 0.01	36.8	p<0.001

Cingulate Cortex	0.17±0.01	0.24±0.01	40.1	p<0.001
Orbito-Frontal Cortex	0.17±0.01	0.23±0.01	36.1	p<0.001
Hippocampus	0.16±0.01	0.21±0.01	34.6	p<0.001
Hypothalamus	0.16±0.01	0.22±0.01	36.6	p<0.001
Insular Cortex	0.18±0.01	0.25±0.01	43.9	p<0.001
Midbrain	0.18±0.01	0.26±0.01	47.8	p<0.001
Occipital Cortex	0.2±0.01	0.27±0.01	32.1	p<0.001
Parietal Cortex	0.18±0.01	0.25±0.01	39	p<0.001
Striatum	0.16±0.01	0.24±0.01	55.3	p<0.001
Temporal Cortex	0.17±0	0.24±0.01	39.6	p<0.001
Thalamus	0.16±0.01	0.26±0.01	60.6	p<0.001
White Matter	0.16±0.01	0.23±0.01	39.4	p<0.001

4. Discussion

The P-gp function at the BBB can be altered by different factors and could affect the distribution of several drugs inside the brain (1,8,17,36,37). PET tracers, such as [^{18}F]MC225, may allow monitoring P-gp function and may identify potential DDIs (13,38–40). This study investigated the suitability of [^{18}F]MC225 as a tracer for measuring P-gp function at the BBB of NHP. The study also explored the use of different methods for quantification of the P-gp function and various PET scan durations.

Blood analysis indicated that 50% of plasma radioactivity represented parent tracer at 30 min after tracer injection. This differs from plasma pharmacokinetics in rats, where only 24% of the parent [^{18}F]MC225 was still available at that time-point (15). This species difference can be expected because the rate of metabolism is smaller in bigger animals. The biological plasma $T_{1/2}$ remained similar after P-gp inhibition, suggesting that tracer elimination from the plasma was not significantly affected by the tariquidar intervention.

According to AIC and SE%, the most appropriate fit was found when vB was fixed to 6% or used as a free fit parameter (free vB). Since restricting vB did not improve the quality of the fits and robustness of the pharmacokinetic parameters, it was decided to use vB as a free fit parameter.

Next, we evaluated the preferred model for each scan duration. Lower AIC values for 2-TCM than for 1-TCM were observed for all studied scan durations. However, SE% V_T with 2-TCM were extremely large for short scan durations and in some cases higher than 1000%. Therefore, we preferred the use of 1-TCM for the analysis of short scans (<30-min) and the use of 2-TCM for long scans (60- and 91-min). The previous validation of [^{18}F]MC225 in rats had also shown unrealistically high V_T for 2-TCM due to very large SE% of K_1-k_4 . Therefore, 1-TCM was selected as the preferred kinetic model for [^{18}F]MC225 in rats (15).

In line with the above discussed previous study, our results showed that the estimation of V_T and k_2 are sensitive to scan duration while K_1 is not (15). For baseline scans, the whole brain V_T increased by 165%, from 4 for the 10-min to 10.6 for the 91-min data. The k_2 also varied inconsistently with scan duration. This behavior is caused by the need to use different kinetic models for short (1-TCM) and long scan durations (2-TCM).

Inhibition of P-gp by a tariquidar challenge increased K_1 and V_T significantly in all the scan durations. These changes were smaller compared with those seen in rats, where the whole brain K_1 was 575% and the V_T 159% higher in tariquidar-treated rats than in control rats (15). For baseline scans, the rat's values were similar to NHP (K_1 (mL/mL/min) NHP = 0.19 vs K_1 rats = 0.12; V_T NHP=9.33 vs V_T rats=7.7). However, after P-gp inhibition the values in rats were higher than the ones in NHP (K_1 NHP = 0.27 vs K_1 rats = 0.81; V_T NHP =13.08 vs V_T rats = 20) (15). These differences may be explained by the dose of tariquidar used in these experiments. Rats were injected i.v. 30 min before the PET scan with a dose of 8 mg/kg which is considered the maximum effective dose in rodents, whereas the NHPs were injected with a dose of 8 mg/kg at 15 min before the PET scan (15,16). In order to reach complete P-gp inhibition in NHP, a higher dose would have been required. Another possibility is that a longer period of time between the tariquidar injection and the PET scan is required to observe a larger P-gp inhibition effect in NHPs.

Taking into account all brain regions, the changes in K_1 between baseline and after-inhibition remained similar for all scan durations tested (42-44%) while the change in V_T varied from 40% using 10-min data to 32% in 91-min scan data. Moreover, K_1 changes caused by the P-gp inhibition were larger than those seen with V_T .

The previous pharmacokinetic evaluation of [^{18}F]MC225 in rats also showed that inhibition with tariquidar caused larger changes in K_1 (6 to 11-fold increase compared with baseline) than in V_T (2 to 4-fold increase) (15). This previous publication and our NHP study support the use of K_1 as a suitable parameter to measure the P-gp function at the BBB (20,21). Since K_1 can be affected by changes in blood flow, we recommend the performance of an additional PET study (e.g. using [^{15}O]H $_2$ O) for measuring the blood flow in order to assess the P-gp function more precisely (20,21).

However, if changes in the blood flow occurred due to the treatment with tariquidar, the k_2 values would have also increased in the after-inhibition scans, because this parameter also possesses information about flow, tracer extraction, and partition coefficient (41). The results of our study did not find any significant increases in the k_2 values after the administration of tariquidar in any of the tested scan durations. It may be argued that the increase in k_2 values due to the blood flow effect may be masked due to the accumulation of radio-metabolites which may decrease the k_2 .

However, in short scan duration (10-, 20- and 30-min), where the interference of the radio-metabolites is negligible, the k_2 values did not change in after-inhibition scans in comparison to baseline values. Additionally, it has been published that the administration of the P-gp inhibitor cyclosporine-A caused changes in the K_1 of [^{11}C]verapamil tracer that cannot be explained by changes in the blood flow. The changes in blood flow, assessed using [^{15}O]H $_2$ O scans, were smaller than the changes observed in the K_1 of [^{11}C]verapamil, thus these changes in K_1 may be mainly caused by the P-gp inhibition (20).

Nevertheless, another approach to measure the P-gp function is to use other flow independent kinetic parameters, such as the V_T . Even though V_T estimations did not become stable at longer scan durations up to 60-min, SE% V_T decreased for scan durations longer than 60-min. Thus, V_T values estimated at scan durations greater than 60-min are more reliable than V_T values estimated from data of shorter scans. Moreover, since P-gp inhibition increased the whole-brain V_T by 40% (for 60-min scans) and by 30% (for 91-min scans), V_T obtained with 2-TCM may be used as a surrogate parameter to measure the P-gp function, but requiring scans longer than 60-min. On the other hand, V_T obtained with long scan durations may be affected by the accumulation of radio-metabolites inside the brain or by unspecific trapping of the tracer, and therefore, these values should be interpreted with caution. The latter may also explain the continued rise of V_T estimations in all tested scan durations.

Recent publications using another weak P-gp tracer, [^{11}C]Metoclopramide, concluded that the most sensitive parameter to measure P-gp inhibition in NHP is V_T . After the infusion of tariquidar for 2 hours at a dose of 4 mg/kg/h, V_T increased 2.2-fold compared to baseline. Despite the differences in dose of tariquidar and route of administration, the investigators observed an increase of 31% of K_1 after-inhibition which is similar to the increase observed in our study. This study also found a significant decrease (1.64-fold) in the efflux rate constant k_2 after P-gp inhibition. The authors suggested that [^{11}C]Metoclopramide may be used to measure altered transporter-mediated efflux reflected in changes of k_2 (42). However, our analysis did not find significant differences in k_2 between baseline and after-inhibition scans. A previous preclinical study performed with [^{18}F]MC225 found an increase in whole-brain k_2 values in rats previously exposed to isoflurane-anesthesia compared to control rats. This increase in k_2 caused a decrease in V_T of the tracer inside the brain, however, it did not correspond to an increase in P-gp brain expression as was confirmed with western blot studies (43). Therefore, if [^{18}F]MC225 is used to measure P-gp function at the BBB, the K_1 may be considered as the best parameter to assess the target function. Future studies such as a head to head comparison of both tracers are needed to clarify the mechanism of action of [^{11}C]Metoclopramide and [^{18}F]MC225 and evaluate their sensitivity and specificity for measuring the P-gp transporter function.

In addition, recently the toxicity effects of MC225 were evaluated in male and female Sprague-Dawley rats. The results of this study indicated that MC225 at a dose of 2.5 mg/kg body weight, which is the 10,000-fold equivalent of the postulated maximum administration dose (0.25 µg/kg body weight), did not cause toxicity to the animals during the following 14 days of observation. Moreover, the *Salmonella* thyphimurium and *Escherichia coli* mutation test did not reveal any mutagenic activity (44). Although the toxicity of MC225 was not evaluated in NHP, these positive results may set the stage for the clinical evaluation of [^{18}F]MC225.

5. Conclusions

This study investigates the use of different kinetic models to assess P-gp function as a function of varying scan durations. For short scan durations, the preferred model to quantify [^{18}F]MC225 PET in NHP is the 1-TCM and the most suitable parameter to estimate the P-gp function is K_1 . For long scan durations (>60-min), 2-TCM is the preferred model and in this case, both K_1 and V_T can be used to estimate P-gp function at the BBB. Overall, our results suggest that the P-gp function at the BBB can be measured using either K_1 obtained by applying 1-TCM to 10-min of scan data or using K_1 and V_T obtained with 2-TCM for 91-min scan duration. These conclusions are in line with previous studies performed in rats and mice and warrant further studies in humans to confirm the properties of [^{18}F]MC225.

6. Supporting Information

Supplemental table 1: Scan data.

[^{18}F]MC225						
Subjects	Scan	Body Weight (kg)	Injected dose (MBq)	Molar activity (GBq/µmol)	Injected mass (nmol)	Hour of injection (hour: min)
Subject 1	Baseline	8.20	778	57.5	13.5	14:47
Subject 1	After-inhibition	8.26	579	35.7	16.2	12:01
Subject 2	Baseline	6.65	699	78.8	8.9	11:56
Subject 2	After-inhibition	6.52	698	65.6	10.6	12:25
Subject 3	Baseline	6.76	671	49.3	13.6	12:45
Subject 3	After-inhibition	7.17	681	59.7	11.4	15:30
Mean±SD* Baseline	-	7.2 ±0.9	716.3±55.6	61.9±15.2	12±2.7	-
Mean±SD* After-inhibition		7.3±0.8	652.5±64.4	53.7±15.8	12.7±3	

* Mean±Standard Deviation (SD).

PET/MRI: Reference MRI and Tissue Probability Maps**Images obtained from:**

- MNI space (Frey): <http://www.bic.mni.mcgill.ca/ServicesAtlases/Macaque>
- Saleem space (McLaren): <http://www.brainmap.wisc.edu/monkey.html>
- CIVM space (Calabrese): <http://www.civm.duhs.duke.edu/rhesusatlas/>

Original files:

- MNI space (Frey et al.):
 - o T1 MRI: macaque_25_model-MNI.mnc
- Saleem space (McLaren et al.)
 - o T1 MRI: 112RM-SL_T1.nii.gz
 - o Tissue probability Maps:
 - gm_priors_ohsu+uw.nii.gz
 - wm_priors_ohsu+uw.nii.gz
 - csf_priors_ohsu+uw.nii.gz
- CIVM space (Calabrese et al.)
 - o b0 MRI: civm_rhesus_v1_b0_downsample2.nii.gz
 - o VOIs:
 - civm_rhesus_v1_labels_downsample2.nii.gz
 - civm_rhesus_v1_label_key.txt
 - civm_rhesus_v1_ontology.xlsx

Final files in MNI space:

- T1 MR: T1_MNI.nii
- T1 brain masked: T1-masked_MNI.nii
- Tissue probability maps: tpm_MNI.nii
- VOIs: CIVM_labels.nii
- Transformation matrices:
 - o From Saleem to MNI space (and inverse):
 - Only affine transformation: SL_2_MNI_affine.mat
 - Including warping: SL_2_MNI_warp.mat
 - o From CIVM space to MNI (and inverse): CIVM_2_MNI.mat

Supplemental table 2: $EMM \pm SE$ of AIC , $SE\%K_1$ and $SE\%V_T$ for all the brain regions at different scan durations at baseline and after-inhibition using different models.

Scan duration	Scan	Model	AIC	SE%K ₁	SE%V _T
			EMM $\pm$ SE	EMM $\pm$ SE	EMM $\pm$ SE
10 min	Baseline	1-tcm	-1 $\pm$ 4.2	2.5 $\pm$ 0.2	10.3 $\pm$ 0.8
		2-tcm	-5 $\pm$ 6.9	8 $\pm$ 0.8	180.9 $\pm$ 42.2
		2-tcm irrev	-8 $\pm$ 6.6	3.9 $\pm$ 0.4	
	Treatment	1-tcm	-12 $\pm$ 4.4	1.9 $\pm$ 0.1	7.8 $\pm$ 0.6
		2-tcm	-20 $\pm$ 2.3	6.9 $\pm$ 2.1	42.1 $\pm$ 5.3
		2-tcm irrev	-22 $\pm$ 3	3.1 $\pm$ 0.2	
20 min	Baseline	1-tcm	1 $\pm$ 3.7	2.1 $\pm$ 0.1	6.8 $\pm$ 0.3
		2-tcm	-17 $\pm$ 8.2	4.8 $\pm$ 0.8	38.3 $\pm$ 3.8
		2-tcm irrev	-12 $\pm$ 6.4	2.8 $\pm$ 0.3	
	Treatment	1-tcm	-14 $\pm$ 5.8	1.7 $\pm$ 0.2	5.2 $\pm$ 0.3
		2-tcm	-39 $\pm$ 2	3.6 $\pm$ 0.4	29.2 $\pm$ 1.9
		2-tcm irrev	-29 $\pm$ 5	2.1 $\pm$ 0.2	
30 min	Baseline	1-tcm	6 $\pm$ 3.3	2 $\pm$ 0.1	5.8 $\pm$ 0.1
		2-tcm	-23 $\pm$ 8.9	4 $\pm$ 0.3	11.8 $\pm$ 0.2
		2-tcm irrev	-12 $\pm$ 6	2.4 $\pm$ 0.2	
	Treatment	1-tcm	-8 $\pm$ 5.3	1.7 $\pm$ 0.2	4.7 $\pm$ 0.3
		2-tcm	-45 $\pm$ 2.7	2.7 $\pm$ 0.2	14.5 $\pm$ 4.4
		2-tcm irrev	-32 $\pm$ 6.8	1.9 $\pm$ 0.2	
60 min	Baseline	1-tcm	20 $\pm$ 4.8	1.8 $\pm$ 0.1	3.5 $\pm$ 0.1
		2-tcm	-34 $\pm$ 9.7	2.7 $\pm$ 0.2	28.7 $\pm$ 13.6
		2-tcm irrev	-14 $\pm$ 4.4	1.9 $\pm$ 0.1	
	Treatment	1-tcm	13 $\pm$ 5.2	1.8 $\pm$ 0.2	3.3 $\pm$ 0.2
		2-tcm	-60 $\pm$ 7.6	2 $\pm$ 0.3	7.1 $\pm$ 1.6
		2-tcm irrev	-37 $\pm$ 6.9	1.6 $\pm$ 0.2	
91 min	Baseline	1-tcm	36 $\pm$ 6	1.9 $\pm$ 0.1	2.7 $\pm$ 0.1
		2-tcm	-46 $\pm$ 9	2.1 $\pm$ 0.1	5.1 $\pm$ 0.6
		2-tcm irrev	-15 $\pm$ 5.1	1.7 $\pm$ 0.1	
	Treatment	1-tcm	24 $\pm$ 6.6	1.7 $\pm$ 0.2	2.5 $\pm$ 0.1
		2-tcm	-85 $\pm$ 10.4	1.7 $\pm$ 0.2	2.5 $\pm$ 0.3
		2-tcm irrev	-27 $\pm$ 6.5	1.5 $\pm$ 0.2	

Supplemental table 3: Mean $\pm$ SE of V_T and $\text{SE}\%V_T$ for all the brain regions in short PET scans using 2-TCM.

Scan duration	Region	Scan			
		Baseline		Treatment	
		Mean $V_T \pm \text{SE}$	Mean $\text{SE}\%V_T \pm \text{SE}$	Mean $V_T \pm \text{SE}$	Mean $\text{SE}\%V_T \pm \text{SE}$
10-min	Basal Ganglia	7.3 $\pm$ 1.67	238.74 $\pm$ 103.53	15.87 $\pm$ 9.75	381.7 $\pm$ 364.09
	Brainstem	10.4 $\pm$ 7.19	182.02 $\pm$ 158.46	1.05E+06 $\pm$ 1.05E+06	2.65E+07 $\pm$ 2.65E+07
	Cerebellum	13.28 $\pm$ 6	90.55 $\pm$ 60.81	10.14 $\pm$ 2.63	37.5 $\pm$ 27.32
	Cingulate Cortex	4.80E+06 $\pm$ 4.80E+06	5.70E+07 $\pm$ 5.70E+07	103.54 $\pm$ 92.38	3014.96 $\pm$ 2960.41
	Orbito-Frontal Cortex	393.94 $\pm$ 385.29	5755.37 $\pm$ 5702.25	10.56 $\pm$ 0.68	38.31 $\pm$ 17.75
	Hippocampus	1.17E+21 $\pm$ 1.17E+21	3.03E+22 $\pm$ 3.03E+22	5.74 $\pm$ 0.21	28.42 $\pm$ 7.93
	Hypothalamus	7.78 $\pm$ 2.53	200.52 $\pm$ 101.28	5049.11 $\pm$ 5040.19	5.09E+05 $\pm$ 5.09E+05
	Insular Cortex	9.47 $\pm$ 3.4	286.24 $\pm$ 192.33	8.05 $\pm$ 0.32	13.32 $\pm$ 0.69
	Midbrain	21.12 $\pm$ 12.86	333.25 $\pm$ 228.2	5.83E+14 $\pm$ 5.83E+14	3.07E+16 $\pm$ 3.07E+16
	Occipital Cortex	157.09 $\pm$ 148.6	2037.84 $\pm$ 1981.01	17.12 $\pm$ 6.85	159.48 $\pm$ 131.15
	Parietal Cortex	45.88 $\pm$ 40.83	1149.83 $\pm$ 1140.36	10.29 $\pm$ 1.66	26.27 $\pm$ 12.71
	Striatum	15.08 $\pm$ 4.37	667.17 $\pm$ 601.52	8.99 $\pm$ 1.61	26.24 $\pm$ 7.79
	Temporal Cortex	11.92 $\pm$ 4.24	210.98 $\pm$ 100.51	6.55 $\pm$ 0.4	13.42 $\pm$ 4.82
	Thalamus	23.67 $\pm$ 11.64	496.53 $\pm$ 243.15	7.9 $\pm$ 0.45	19.97 $\pm$ 6.57
	White Matter	10.39 $\pm$ 4.13	108.24 $\pm$ 77.03	1.30E+04 $\pm$ 1.30E+04	3.50E+05 $\pm$ 3.50E+05
	Whole Brain	2256.19 $\pm$ 2249.6	52385.12 $\pm$ 52346.37	8.26 $\pm$ 0.97	15.09 $\pm$ 5.32
20-min	Basal Ganglia	7.11 $\pm$ 1.57	38.6 $\pm$ 4.63	12.01 $\pm$ 3.14	71.7 $\pm$ 52.51
	Brainstem	10.9 $\pm$ 6	80.32 $\pm$ 63.71	7.8 $\pm$ 0.42	17.2 $\pm$ 4.68
	Cerebellum	8.78 $\pm$ 0.96	12.7 $\pm$ 5.26	9 $\pm$ 0.53	5.47 $\pm$ 1.41
	Cingulate Cortex	9.33 $\pm$ 1.04	35.1 $\pm$ 18.12	11.83 $\pm$ 1.82	27.1 $\pm$ 12.54
	Orbito-Frontal Cortex	7.86 $\pm$ 1.28	13.37 $\pm$ 2.95	8.65 $\pm$ 0.33	3.93 $\pm$ 0.99
	Hippocampus	7.67 $\pm$ 0.65	36.72 $\pm$ 11.26	8.07E+10 $\pm$ 8.07E+10	4.33E+12 $\pm$ 4.33E+12
	Hypothalamus	6.81 $\pm$ 1.65	96.69 $\pm$ 66.89	16.14 $\pm$ 7.56	158.08 $\pm$ 128.02

	Insular Cortex	11.48 ± 0.02	77.75 ± 21.05	668.22 ± 659.07	71948.99 ± 71943.27
	Midbrain	10.61 ± 3.35	63.41 ± 31.38	79.19 ± 69.42	2388.57 ± 2374.72
	Occipital Cortex	7.07 ± 0.59	5.63 ± 1.43	9 ± 0.33	5 ± 0.83
	Parietal Cortex	7.76 ± 0.16	28.63 ± 22.84	12.91 ± 4.06	35.13 ± 31.56
	Striatum	7.37 ± 1.09	39.47 ± 30.91	9.45 ± 0.32	11.16 ± 4.58
	Temporal Cortex	9.16 ± 0.47	52.48 ± 32.18	8.93 ± 0.54	6.19 ± 1.75
	Thalamus	6.49 ± 0.72	12.24 ± 1.52	13.02 ± 3.95	50.54 ± 39.94
	White Matter	6.81 ± 0.82	8.3 ± 1.43	8.32 ± 0.5	3.54 ± 0.43
	Whole Brain	8.33 ± 1.61	11.24 ± 3.04	8.64 ± 0.19	3.43 ± 0.18
30-min	Basal Ganglia	4.30E+08 ± 4.30E+08	1.15E+10 ± 1.15E+10	10.77 ± 1	17.89 ± 7.11
	Brainstem	2.25E+46 ± 2.25E+46	3E+49 ± 3E+49	8.58 ± 0.48	10.99 ± 2.41
	Cerebellum	9.24 ± 1.36	6.08 ± 2.13	9.73 ± 0.47	3.46 ± 0.48
	Cingulate Cortex	8.25 ± 0.76	9.12 ± 3.02	11.09 ± 0.92	10.47 ± 4.58
	Orbito-Frontal Cortex	8.08 ± 1.07	5.99 ± 1.2	9.56 ± 0.57	2.78 ± 0.47
	Hippocampus	7.36 ± 0.94	13.99 ± 1.14	46.92 ± 29.73	986.97 ± 873.6
	Hypothalamus	7.5 ± 0.62	29.75 ± 4	10.54 ± 0.61	25.95 ± 6.94
	Insular Cortex	9.26 ± 1.04	31.9 ± 11.36	15.92 ± 0.86	39.39 ± 5.02
	Midbrain	7.45 ± 0.49	12.5 ± 2.53	10.75 ± 0.4	8.5 ± 1.66
	Occipital Cortex	7.47 ± 0.54	3.39 ± 0.72	9.64 ± 0.49	3.25 ± 0.21
	Parietal Cortex	7.47 ± 0.59	3.88 ± 0.87	9.9 ± 0.39	2.52 ± 0.34
	Striatum	39.93 ± 32.56	3717.5 ± 3705.21	10.3 ± 0.74	7.39 ± 3.18
	Temporal Cortex	8.49 ± 0.7	12.43 ± 5.96	10.12 ± 0.9	4.66 ± 1.27
	Thalamus	7.15 ± 0.77	13.67 ± 6.3	9.85 ± 0.38	5.98 ± 0.49
	White Matter	6.88 ± 0.59	3.89 ± 1.06	9.32 ± 0.56	2.69 ± 0.19
	Whole Brain	7.93 ± 0.97	4.4 ± 0.95	9.93 ± 0.71	3.38 ± 0.85

Supplemental table 4: $\text{EMM} \pm \text{SE}$ of V_T at baseline and after-inhibition in short scan durations for all the regions and p values of the difference between baseline and after-inhibition scans.

Scan duration	Region	V_T baseline $\pm$ SE	V_T after-inhibition $\pm$ SE	% Change V_T	p value
10min	Basal Ganglia	3.7 $\pm$ 0.3	5.2 $\pm$ 0.3	40.9	p=0.01
10min	Brainstem	3.5 $\pm$ 0.2	4.3 $\pm$ 0.4	24.1	p=0.2
10min	Cerebellum	4.7 $\pm$ 0.6	5.7 $\pm$ 0.3	20.9	p=0.251
10min	Cingulate Cortex	4.1 $\pm$ 0.2	6 $\pm$ 0.3	45.2	p<0.001
10min	Orbito-Frontal Cortex	3.8 $\pm$ 0.2	5.5 $\pm$ 0.4	43.4	p=0.003
10min	Hippocampus	3.6 $\pm$ 0.6	5 $\pm$ 0.3	38.3	p=0.024
10min	Hypothalamus	3.9 $\pm$ 0.5	4.5 $\pm$ 0.6	15.8	p=0.577
10min	Insular Cortex	3.7 $\pm$ 0.1	6.5 $\pm$ 0.3	76.3	p<0.001
10min	Midbrain	3.8 $\pm$ 0.4	6 $\pm$ 0.4	56.3	p<0.001
10min	Occipital Cortex	4.5 $\pm$ 0.3	6.2 $\pm$ 0.4	38.0	p=0.003
10min	Parietal Cortex	4.1 $\pm$ 0.1	5.9 $\pm$ 0.3	46.1	p<0.001
10min	Striatum	4.1 $\pm$ 0.1	5.6 $\pm$ 0.4	35.4	p=0.001
10min	Temporal Cortex	4.1 $\pm$ 0.2	5.5 $\pm$ 0.3	33.4	p=0.004
10min	Thalamus	3.7 $\pm$ 0.2	5.8 $\pm$ 0.6	55.0	p=0.013
10min	White Matter	3.7 $\pm$ 0.3	5.3 $\pm$ 0.2	41.7	p<0.001
10min	Whole Brain	4 $\pm$ 0.3	5.5 $\pm$ 0.3	38.8	p=0.004
20 min	Basal Ganglia	4.6 $\pm$ 0.3	6.8 $\pm$ 0.2	49.7	p<0.001
20 min	Brainstem	4.8 $\pm$ 0.6	5.6 $\pm$ 0.3	16.8	p=0.302
20 min	Cerebellum	6.5 $\pm$ 0.8	7.3 $\pm$ 0.3	12.0	p=0.415
20 min	Cingulate Cortex	5.6 $\pm$ 0.4	7.7 $\pm$ 0.2	37.6	p<0.001
20 min	Orbito-Frontal Cortex	5 $\pm$ 0.3	6.9 $\pm$ 0.3	37.3	p=0.002
20 min	Hippocampus	5.2 $\pm$ 0.5	6.3 $\pm$ 0.3	19.7	p=0.001
20 min	Hypothalamus	4.8 $\pm$ 0.5	6.2 $\pm$ 0.5	29.1	p=0.111
20 min	Insular Cortex	5.2 $\pm$ 0.1	8 $\pm$ 0.3	54.4	p<0.001
20 min	Midbrain	5.3 $\pm$ 0.5	7.5 $\pm$ 0.2	41.5	p<0.001
20 min	Occipital Cortex	5.7 $\pm$ 0.3	7.5 $\pm$ 0.2	31.0	p<0.001
20 min	Parietal Cortex	5.4 $\pm$ 0.2	7.5 $\pm$ 0.3	39.6	p<0.001
20 min	Striatum	4.9 $\pm$ 0.4	7.2 $\pm$ 0.2	45.2	p<0.001
20 min	Temporal Cortex	5.4 $\pm$ 0.4	7.1 $\pm$ 0.3	32.8	p=0.004
20 min	Thalamus	5 $\pm$ 0.4	7.3 $\pm$ 0.4	46.9	p=0.003
20 min	White Matter	4.9 $\pm$ 0.3	6.7 $\pm$ 0.2	37.2	p<0.001
20 min	Whole Brain	5.3 $\pm$ 0.4	7 $\pm$ 0.2	33.1	p=0.003
30 min	Basal Ganglia	5.5 $\pm$ 0.4	7.9 $\pm$ 0.2	45.0	p<0.001
30 min	Brainstem	5.5 $\pm$ 0.7	6.4 $\pm$ 0.3	15.9	p=0.321
30 min	Cerebellum	7.5 $\pm$ 0.9	8.3 $\pm$ 0.3	11.0	p=0.419
30 min	Cingulate Cortex	6.4 $\pm$ 0.5	8.7 $\pm$ 0.2	36.5	p<0.001
30 min	Orbito-Frontal Cortex	5.8 $\pm$ 0.3	8 $\pm$ 0.3	36.0	p=0.001
30 min	Hippocampus	6 $\pm$ 0.6	7.5 $\pm$ 0.3	24.1	p<0.001
30 min	Hypothalamus	5.7 $\pm$ 0.4	7.4 $\pm$ 0.3	29.5	p=0.013
30 min	Insular Cortex	6 $\pm$ 0.1	9.2 $\pm$ 0.4	54.1	p<0.001
30 min	Midbrain	5.9 $\pm$ 0.4	8.7 $\pm$ 0.2	46.3	p<0.001
30 min	Occipital Cortex	6.4 $\pm$ 0.4	8.4 $\pm$ 0.2	30.4	p<0.001
30 min	Parietal Cortex	6.1 $\pm$ 0.3	8.5 $\pm$ 0.3	39.5	p<0.001
30 min	Striatum	5.5 $\pm$ 0.4	8.2 $\pm$ 0.2	48.1	p<0.001
30 min	Temporal Cortex	6.2 $\pm$ 0.5	8.2 $\pm$ 0.3	31.8	p=0.003
30 min	Thalamus	5.7 $\pm$ 0.4	8.2 $\pm$ 0.3	45.9	p<0.001
30 min	White Matter	5.6 $\pm$ 0.4	7.7 $\pm$ 0.2	37.3	p<0.001
30 min	Whole Brain	6 $\pm$ 0.4	8 $\pm$ 0.2	33.0	p=0.001

Supplemental table 5: EMM± SE of V_T at baseline and after-inhibition in long scan durations for all the regions and p values of the difference between baseline and after-inhibition scans.

Scan duration	Region	V_T baseline±SE	V_T after-inhibition±SE	% Change V_T	p value
60 min	Basal Ganglia	9.6±0.6	14.1±0.8	46.8	p<0.001
60 min	Brainstem	11.7±1.9	12.7±0.8	9.2	p=0.596
60 min	Cerebellum	10.7±0.8	13.2±0.1	23.8	p=0.002
60 min	Cingulate Cortex	9.6±0.4	14.1±0.7	47.6	p<0.001
60 min	Orbito-Frontal Cortex	9.3±0.6	13.4±0.5	43.4	p<0.001
60 min	Hippocampus	9.8±1	14.5±0.9	48.0	p<0.001
60 min	Hypothalamus	22±10.1	15.5±1.1	-29.6	p=0.552
60 min	Insular Cortex	9.6±0.8	16.2±0.8	69.8	p<0.001
60 min	Midbrain	9.4±0.7	14.9±1.1	58.5	p<0.001
60 min	Occipital Cortex	9.1±0.4	12.7±0.4	39.2	p<0.001
60 min	Parietal Cortex	8.9±0.5	13.3±0.5	49.9	p<0.001
60 min	Striatum	9.5±0.6	12.9±0.4	35.6	p<0.001
60 min	Temporal Cortex	9.7±0.5	13.7±0.4	40.4	p<0.001
60 min	Thalamus	8.2±0.6	14.6±1.6	78.7	p=0.001
60 min	White Matter	8.7±0.6	12.6±0.4	46.1	p<0.001
60 min	Whole Brain	9.3±0.5	13.1±0.4	40.3	p<0.001
91 min	Basal Ganglia	10.8±0.6	15.1±0.5	40.3	p<0.001
91 min	Brainstem	10.8±1.1	13.1±0.4	21.9	p=0.111
91 min	Cerebellum	12±1.1	13.7±0.2	14.3	p=0.122
91 min	Cingulate Cortex	10.8±0.4	14.9±0.2	37.3	p<0.001
91 min	Orbito-Frontal Cortex	10.7±0.4	14.1±0.3	31.4	p<0.001
91 min	Hippocampus	11.5±0.8	15.3±0.7	33.1	p<0.001
91 min	Hypothalamus	13.4±0.9	15.6±0.4	16.7	p=0.086
91 min	Insular Cortex	10.9±0.3	15.9±0.3	46.3	p<0.001
91 min	Midbrain	10.7±0.6	14.9±0.4	40.1	p<0.001
91 min	Occipital Cortex	10.5±0.4	13.1±0.2	25.4	p<0.001
91 min	Parietal Cortex	10.5±0.4	13.7±0.2	30.8	p<0.001
91 min	Striatum	10.2±0.4	13.8±0.3	35.4	p<0.001
91 min	Temporal Cortex	11±0.5	14.6±0.1	31.8	p<0.001
91 min	Thalamus	9.7±0.6	13.9±0.2	42.3	p<0.001
91 min	White Matter	10.1±0.5	13.5±0.1	34.1	p<0.001
91 min	Whole Brain	10.6±0.5	13.8±0.1	30.4	p<0.001

Supplemental table 6: EMM $\pm$ SE of K_1 at baseline and after-inhibition in short scan durations for all the regions and p values of the difference between baseline and after-inhibition scans.

Scan duration	Region	K_1 baseline $\pm$ SE	K_1 after-inhibition $\pm$ SE	% Change K_1	p value
10min	Basal Ganglia	0.16 $\pm$ 0.01	0.24 $\pm$ 0.02	45.5	p<0.001
10min	Brainstem	0.15 $\pm$ 0.01	0.23 $\pm$ 0.01	51.6	p<0.001
10min	Cerebellum	0.21 $\pm$ 0.01	0.29 $\pm$ 0.01	36.2	p<0.001
10min	Cingulate Cortex	0.19 $\pm$ 0.01	0.26 $\pm$ 0.01	39.1	p<0.001
10min	Orbito-Frontal Cortex	0.18 $\pm$ 0.01	0.25 $\pm$ 0.01	34.7	p<0.001
10min	Hippocampus	0.17 $\pm$ 0.01	0.23 $\pm$ 0.01	33.6	p<0.001
10min	Hypothalamus	0.17 $\pm$ 0.01	0.24 $\pm$ 0.02	41.6	p<0.001
10min	Insular Cortex	0.19 $\pm$ 0.01	0.27 $\pm$ 0.01	39.3	p<0.001
10min	Midbrain	0.19 $\pm$ 0.01	0.28 $\pm$ 0.01	46.2	p<0.001
10min	Occipital Cortex	0.21 $\pm$ 0.01	0.28 $\pm$ 0.01	31.2	p<0.001
10min	Parietal Cortex	0.19 $\pm$ 0.01	0.26 $\pm$ 0.01	38	p<0.001
10min	Striatum	0.17 $\pm$ 0.01	0.26 $\pm$ 0.01	58.8	p<0.001
10min	Temporal Cortex	0.19 $\pm$ 0	0.26 $\pm$ 0.02	40.2	p<0.001
10min	Thalamus	0.17 $\pm$ 0.01	0.27 $\pm$ 0.01	60.6	p<0.001
10min	White Matter	0.17 $\pm$ 0.01	0.24 $\pm$ 0.01	38.7	p<0.001
10min	Whole Brain	0.19 $\pm$ 0.01	0.26 $\pm$ 0.01	38.7	p<0.001
20 min	Basal Ganglia	0.16 $\pm$ 0.01	0.23 $\pm$ 0.01	44.1	p<0.001
20 min	Brainstem	0.14 $\pm$ 0.01	0.22 $\pm$ 0.01	50.2	p<0.001
20 min	Cerebellum	0.2 $\pm$ 0.01	0.27 $\pm$ 0.01	37.2	p<0.001
20 min	Cingulate Cortex	0.18 $\pm$ 0.01	0.25 $\pm$ 0.01	39.7	p<0.001
20 min	Orbito-Frontal Cortex	0.17 $\pm$ 0.01	0.24 $\pm$ 0.01	35.8	p<0.001
20 min	Hippocampus	0.16 $\pm$ 0.01	0.22 $\pm$ 0.01	35.9	p<0.001
20 min	Hypothalamus	0.16 $\pm$ 0.01	0.23 $\pm$ 0.01	37.1	p<0.001
20 min	Insular Cortex	0.18 $\pm$ 0.01	0.26 $\pm$ 0.01	43.3	p<0.001
20 min	Midbrain	0.18 $\pm$ 0.01	0.27 $\pm$ 0.01	48.6	p<0.001
20 min	Occipital Cortex	0.21 $\pm$ 0.01	0.27 $\pm$ 0.01	32.1	p<0.001
20 min	Parietal Cortex	0.18 $\pm$ 0.01	0.25 $\pm$ 0.01	39	p<0.001
20 min	Striatum	0.16 $\pm$ 0.01	0.25 $\pm$ 0.01	56.1	p<0.001
20 min	Temporal Cortex	0.18 $\pm$ 0	0.25 $\pm$ 0.01	39.8	p<0.001
20 min	Thalamus	0.16 $\pm$ 0.01	0.26 $\pm$ 0.01	60.6	p<0.001
20 min	White Matter	0.17 $\pm$ 0.01	0.23 $\pm$ 0.01	39.4	p<0.001
20 min	Whole Brain	0.18 $\pm$ 0.01	0.25 $\pm$ 0.01	39.3	p<0.001
30 min	Basal Ganglia	0.15 $\pm$ 0.01	0.22 $\pm$ 0.01	44.9	p<0.001
30 min	Brainstem	0.14 $\pm$ 0.01	0.21 $\pm$ 0.01	49	p<0.001
30 min	Cerebellum	0.19 $\pm$ 0.01	0.27 $\pm$ 0.01	36.8	p<0.001
30 min	Cingulate Cortex	0.17 $\pm$ 0.01	0.24 $\pm$ 0.01	40.1	p<0.001
30 min	Orbito-Frontal Cortex	0.17 $\pm$ 0.01	0.23 $\pm$ 0.01	36.1	p<0.001
30 min	Hippocampus	0.16 $\pm$ 0.01	0.21 $\pm$ 0.01	34.6	p<0.001
30 min	Hypothalamus	0.16 $\pm$ 0.01	0.22 $\pm$ 0.01	36.6	p<0.001
30 min	Insular Cortex	0.18 $\pm$ 0.01	0.25 $\pm$ 0.01	43.9	p<0.001
30 min	Midbrain	0.18 $\pm$ 0.01	0.26 $\pm$ 0.01	47.8	p<0.001
30 min	Occipital Cortex	0.2 $\pm$ 0.01	0.27 $\pm$ 0.01	32.1	p<0.001
30 min	Parietal Cortex	0.18 $\pm$ 0.01	0.25 $\pm$ 0.01	39	p<0.001
30 min	Striatum	0.16 $\pm$ 0.01	0.24 $\pm$ 0.01	55.3	p<0.001
30 min	Temporal Cortex	0.17 $\pm$ 0	0.24 $\pm$ 0.01	39.6	p<0.001
30 min	Thalamus	0.16 $\pm$ 0.01	0.26 $\pm$ 0.01	60.6	p<0.001
30 min	White Matter	0.16 $\pm$ 0.01	0.23 $\pm$ 0.01	39.4	p<0.001
30 min	Whole Brain	0.17 $\pm$ 0.01	0.24 $\pm$ 0.01	39.1	p<0.001

Supplemental table 7: EMM± SE of K_1 at baseline and after-inhibition in long scan durations for all the regions and p values of the difference between baseline and after-inhibition scans.

Scan duration	Region	K_1 baseline±SE	K_1 after-inhibition±SE	% Change K_1	p value
60 min	Basal Ganglia	0.17±0.01	0.25±0.03	49.2	p=0.001
60 min	Brainstem	0.16±0.02	0.25±0.02	51.5	p<0.001
60 min	Cerebellum	0.22±0.02	0.3±0.01	35.2	p<0.001
60 min	Cingulate Cortex	0.2±0.01	0.27±0.01	36.9	p<0.001
60 min	Orbito-Frontal Cortex	0.19±0.01	0.25±0.01	33.2	p=0.001
60 min	Hippocampus	0.18±0.01	0.24±0.02	33.5	p<0.001
60 min	Hypothalamus	0.18±0.01	0.26±0.02	45.7	p<0.001
60 min	Insular Cortex	0.2±0.01	0.27±0	36.2	p<0.001
60 min	Midbrain	0.2±0.01	0.29±0.01	46.2	p<0.001
60 min	Occipital Cortex	0.23±0.01	0.29±0.01	28.3	p<0.001
60 min	Parietal Cortex	0.2±0.01	0.27±0.01	35.9	p=0.001
60 min	Striatum	0.17±0.01	0.28±0.02	64	p<0.001
60 min	Temporal Cortex	0.19±0	0.27±0.02	40.6	p<0.001
60 min	Thalamus	0.19±0.01	0.29±0.02	51.2	p<0.001
60 min	White Matter	0.18±0.01	0.25±0.01	38.5	p<0.001
60 min	Whole Brain	0.19±0.01	0.27±0.01	39	p<0.001
91 min	Basal Ganglia	0.17±0.01	0.25±0.02	46.9	p<0.001
91 min	Brainstem	0.16±0.01	0.24±0.02	56.4	p<0.001
91 min	Cerebellum	0.21±0.02	0.3±0.01	37.8	p<0.001
91 min	Cingulate Cortex	0.2±0.01	0.27±0.01	37	p<0.001
91 min	Orbito-Frontal Cortex	0.19±0.01	0.25±0.01	36	p<0.001
91 min	Hippocampus	0.18±0.02	0.23±0.02	30.5	p<0.001
91 min	Hypothalamus	0.17±0.01	0.26±0.02	50.3	p<0.001
91 min	Insular Cortex	0.2±0.01	0.27±0	41	p<0.001
91 min	Midbrain	0.2±0.01	0.29±0.01	47.5	p<0.001
91 min	Occipital Cortex	0.22±0.01	0.29±0.01	33.1	p<0.001
91 min	Parietal Cortex	0.19±0.01	0.27±0.01	40.2	p<0.001
91 min	Striatum	0.17±0.01	0.27±0.02	61.2	p<0.001
91 min	Temporal Cortex	0.19±0	0.27±0.02	41.6	p<0.001
91 min	Thalamus	0.17±0.01	0.29±0.02	64.7	p<0.001
91 min	White Matter	0.18±0.01	0.25±0.01	40.4	p<0.001
91 min	Whole Brain	0.19±0.01	0.26±0.01	40.7	p<0.001

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Chapter 5

Pharmacokinetic modelling of (*R*)-[¹¹C]verapamil to measure the P-glycoprotein function in non- human primates

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Abstract

(*R*)-[^{11}C]verapamil is a radiotracer widely used for the evaluation of the P-glycoprotein (P-gp) function at the blood-brain barrier (BBB). Several studies have evaluated the pharmacokinetics of (*R*)-[^{11}C]verapamil in rats and humans under different conditions. However, to the best of our knowledge, the pharmacokinetics of (*R*)-[^{11}C]verapamil have not yet been evaluated in non-human primates. Our study aims to establish (*R*)-[^{11}C]verapamil as a reference P-gp tracer for comparison of newly developed P-gp PET tracer in a species close to humans. Therefore, the study assesses the kinetics of (*R*)-[^{11}C]verapamil and evaluates the effect of the scan duration and P-gp inhibition on estimated pharmacokinetic parameters. Three non-human primates underwent two dynamic 91-min Positron Emission Tomography (PET) scans with arterial blood sampling, one at baseline and another after inhibition of the P-gp function. (*R*)-[^{11}C]verapamil data were analyzed using 1-Tissue-Compartment Model (1-TCM) and 2-Tissue-Compartment Model (2-TCM) fits using plasma corrected for polar radio-metabolites or non-corrected for radio-metabolites as input function, and with various scan durations (10-, 20-, 30-, 60- and 91-min). The preferred model was chosen according to the Akaike Information Criterion (AIC) and the standard errors (SE%) of the estimated parameters. The 1-TCM was selected as the model of choice to analyze the (*R*)-[^{11}C]verapamil data at baseline and after inhibition and for all scan durations tested. The volume of distribution (V_T) and efflux constant k_2 estimations were affected by the evaluated scan durations whereas the influx constant K_1 estimations remained relatively constant. After P-gp inhibition (tariquidar, 8 mg/kg), in a 91-min scan duration, the whole-brain V_T increased significantly up to 208% ($p < 0.001$) and K_1 up to 159% ($p < 0.001$) compared with baseline scans. The k_2 values decreased significantly after P-gp inhibition in all the scan durations except for the 91-min scans. This study suggests the use of K_1 , calculated with 1-TCM and using short PET scans (10- to 30-min), as a suitable parameter to measure the P-gp function at the BBB of non-human primates.

Keywords: ABC-transporters, Brain imaging, Efflux transporters, Kinetics, P-gp tracers, Rhesus monkeys.

1. Introduction

(*R*)-[^{11}C]verapamil is a radiotracer used to assess the P-glycoprotein (P-gp) function at the blood-brain barrier (BBB). Verapamil is a calcium channel blocker that is used in the treatment of cardiovascular diseases such as hypertension and arrhythmias (1), and it is also characterized as a substrate for the efflux transporter P-gp. Therefore, [^{11}C]verapamil has been used to assess the P-gp function in the human brain by Positron Emission Tomography (PET) (2,3).

P-gp is a transmembrane protein that belongs to the ATP Binding Cassette (ABC) transporter family. This transporter uses the energy provided by hydrolysis of ATP to move endogenous and exogenous compounds across the membranes (4). The P-gp transporter at the BBB is expressed at the luminal side of the endothelial cells where it pumps a wide variety of substances from the brain to the blood, protecting the central nervous system (CNS) from harmful damage (5). P-gp dysfunctions have been related to the onset and progression of several neurodegenerative diseases and psychiatric disorders (6–8). Furthermore, numerous unrelated CNS drugs are P-gp substrates and, therefore, dysfunction in the P-gp transporter can alter the concentration of these drugs in the brain causing toxicity issues or decreases in drug efficacy (9,10). Thus, it is of interest to evaluate the P-gp function *in vivo* with PET imaging, which is a non-invasive technique and an excellent tool for quantification of biological processes (11–13). The P-gp function was evaluated for the first time in 1996 using a racemic mixture of [^{11}C]verapamil (2) and since then the P-gp function has been extensively studied in man with this tracer (7,8,14–20).

[^{11}C]verapamil was first prepared and evaluated as a racemic mixture (2). However, the (*R*) and (*S*)-verapamil enantiomers showed differences in the rate of metabolism, affinity to calcium channels, plasma protein binding, and clearance that complicate the pharmacokinetic quantification of the racemic tracer (14,21). Therefore, one of the enantiomers had to be selected for labeling. As (*R*)-[^{11}C]verapamil showed less radio-metabolites and lower affinity to calcium channels than (*S*)-[^{11}C]verapamil, it was selected as the most adequate tracer for measuring P-gp function (21).

Since then, several studies have reported the ability of (*R*)-[^{11}C]verapamil to measure the P-gp function (13). For instance, after the administration of a P-gp inhibitor, the P-gp function is decreased and, consequently, the radiotracer is accumulated inside the brain (22–24). This tracer has a very high affinity for the P-gp transporter and, therefore, at baseline conditions, when the P-gp transporter is working adequately, the tracer uptake is very low because the P-gp transporter pumps most of the tracer out of the brain (11,25). Novel PET tracers with lower P-gp affinity have been developed to achieve higher tracer uptake in the brain at baseline conditions (10,11).

The development of new P-gp tracers that have lower affinity to the P-gp transporter (weak P-gp tracers) (26,27) requires kinetic comparison with (R)-[¹¹C]verapamil, which is considered as the reference P-gp PET tracer because its kinetics have been widely evaluated in rats and human under different conditions (16,28–30). This comparison may lead to a greater understanding of the kinetic properties of weak and avid substrates. Weak P-gp tracers, such as [¹¹C]metoclopramide and [¹⁸F]MC225, have been evaluated in rats and also in non-human primates that are more closely related to humans biologically (26,27,31). However, to perform a head-to-head comparison in non-human primates, a kinetic modeling evaluation of (R)-[¹¹C]verapamil needs to be performed in monkeys first. So far, only the kinetics of racemic [¹¹C]verapamil were evaluated (32–34). One study has assessed the kinetics of (R)-[¹¹C]verapamil in rhesus monkeys, but it did not provide a full kinetic evaluation of the tracer (35).

This study aims to assess the kinetics of (R)-[¹¹C]verapamil tracer at baseline and after the inhibition of the P-gp function, using different scan durations (10-, 20-, 30-, 60- and 91-min), and exploring different compartmental models. Moreover, the paper aims to determine the most suitable and reliable parameter to measure the P-gp function (and its change) at the BBB of non-human primates.

2. Experimental Section

2.1. Chemicals and Synthesis of the tracer

Tariquidar (MedChemExpress, New Jersey, USA) was used as P-gp inhibitor (24,29,36). Tariquidar solution for intravenous injection was prepared as previously described (37), with 11 mg of tariquidar methanesulfonate hydrate suspended in 3 ml of saline solution and the injection volume was adjusted to the weight of each monkey (8 mg/kg of body weight). The radiosynthesis and the quality control of (R)-[¹¹C]verapamil were performed as described previously (21).

2.2. Animals

Three healthy male rhesus monkeys (*Macaca mulatta*; Hamri Co. Ltd., Ibaraki, Japan) were included in this study that was performed at the Central Research Laboratory, Hamamatsu Photonics (Hamamatsu, Japan) in collaboration with the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). The animals were housed individually in a controlled environment (24 ± 4 °C, 50 ± 20% of humidity under a 14-hour light/10-hour dark cycle), and maintained and handled in strict accordance with the recommendations of the National Institute of Health and the guidelines of the Ethics Committee of the Central Research Laboratory, Hamamatsu Photonics

(approval HPK-2016-07A), and the Institutional Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology (approval 16067).

2.3. Data collection

2.3.1. Experimental design

Each non-human primate underwent two PET scans with (*R*)-[^{11}C]verapamil. One was performed at baseline conditions and the second one after the inhibition of the P-gp function with tariquidar. The time interval between the scans was 2 hours except for one animal where it was 2 months due to technical issues. Tariquidar was slowly injected through a catheter inserted into a saphenous vein 15 minutes before the PET scans at a dose of 8 mg/kg of body weight. Table 1 shows the body weight of the monkeys on the scanning day, the day of the scan, and hour of radiotracer injection.

Table 1: Design of the experiments.

(<i>R</i>)-[^{11}C]verapamil				
Non-human primates	Scan	Experiment date dd/mm/yyyy	Hour of injection (hour: min)	body weight (kg)
Subject 1	Baseline	27/12/2016	10:59	8.2
Subject 1	After-inhibition	27/12/2016	14:45	8.2
Subject 2	Baseline	17/02/2017	11:01	6.1
subject 2	After-inhibition	17/02/2017	14:03	6.1
Subject 3	Baseline	28/02/2017	12:45	7.2
Subject 3	After-inhibition	26/12/2016	14:01	6.6

2.2.2. PET Imaging acquisition and Reconstruction

The same acquisition protocol was used in a previous study (37). Briefly, one week before the first PET scan, a brain T1 weighted MRI scan of the animal was made (Signa Excite HDTx 3.0T, GE Healthcare) (38). Brain PET scans were acquired using a high-resolution animal PET scanner (SHR-38000, Hamamatsu Photonics). All non-human primates underwent dynamic PET scans with arterial blood sampling. Monkeys were anesthetized (2.5% sevoflurane) during the arterial cannulation and their transport, but they were awake during the scans and the head was immobilized using a fixation device. The animals were positioned in the camera in a sitting position with stereotactic coordinates aligned paralleled to the orbit meatal plane.

Prior to tracer injection, a rotating $^{68}\text{Ge}/^{68}\text{Ga}$ rod source was used to perform the transmission scan (60 min), and its information was used for attenuation correction of the PET images. Next,

animals were injected with (R)-[¹¹C]verapamil (more information in Table 2) at the start of the emission scan (91-min), via the saphenous vein over a period of 30 seconds as a single bolus.

PET images were reconstructed using the Filtered Back Projection method with a Hanning filter of 4.5 mm in SHR-38000 Reconstruction software (Hamamatsu Photonics) and were composed of 49 frames (6 x 10, 6 x 30, 12 x 60, and 25 x 180 s).

2.2.3. Arterial Blood Sampling

As has been described before (37), after the administration of the tracer, 19 blood samples (0.5 ml) were drawn from a cannula placed in the posterior tibial artery at selected time points (8, 16, 24, 32, 40, 48, 56, 64 seconds and 1.5, 2.5, 4, 6, 10, 20, 30, 45, 60, 75, 90 minutes). Then, plasma and blood were separated by centrifugation (12,000 rpm, 60 s) and the radioactivity was measured using a gamma-counter (1480 Wizard, PerkinElmer). The total amount of blood removed from the animal was less than 35 mL/day in each animal.

2.2.4. Metabolite Analysis

Parent fraction and polar radioactive metabolites of (R)-[¹¹C]verapamil in arterial blood samples were determined following tracer injection (16, 40, 64 seconds and 6, 10, 30, 45, 60, 75, 90 minutes). A 0.2 ml volume of ethanol was added to the 0.1 ml of plasma fraction to deproteinize samples. After centrifugation (13,400 rpm, 12,100xg, for 90 s), the samples were analyzed using thin-layer chromatography plates (silica gel 60 F254, Merk) with a mobile phase of ethyl acetate/trimethylamine (9/1). The parent ($R_f = 0.55$) and polar metabolized fraction ($R_f = 0.0$, origin) were assessed using a phosphor imaging plate and a bioimaging analyzer (FLA-7000, Fuji Film).

The percentage of the polar radio-metabolites in plasma was calculated for each animal by fitting a single exponential equation to the values obtained from the metabolite analysis, using an iterative non-linear least-squares approach (GraphPad Prism version 7.02, California, USA): $Y = Y_0 * \exp(-K_e * X)$ (39). Where Y is the percentage of parent tracer at different time points, Y_0 is the intercept, K_e is the first-order elimination constant, and X the time.

2.4. Analysis of PET data

2.4.1. Time-Activity Curves of Blood Sampling: Input function

Time-activity curves (TAC) were determined using the activity of the whole-blood and plasma and expressed as Standardized Uptake Value (SUV):

$$SUV = \text{radioactive concentration} / (\text{injected dose} / \text{body weight}).$$

The radioactivity measured in blood samples was corrected for decay from the time point of tracer administration. The plasma SUV-TAC corrected for polar radio-metabolites (i.e. metabolite-corrected plasma) was calculated multiplying the SUV values of plasma samples by the percentage of parent tracer.

A single exponential equation was fitted to the radio-metabolite-corrected SUV-TAC individually, using the values after the peak (from 360 to 5400 seconds), by an iterative non-linear least-squares approach (GraphPad Prism version 7.02, California, USA): $Y = Y_0 * \exp(-K_e * X)$. Y represents the SUV values at different time points, Y₀ is the intercept, K_e is the first-order elimination constant, and X the time. This approach allows the determination of the rate of tracer elimination (K_e) and the biological half-life (T_{1/2}): $T_{1/2} = \ln(2)/K_e$ (40).

2.4.2. PET/MRI analysis

Images were processed using PMOD v3.8 software (PMOD Technologies, Zürich, Switzerland). All the scans were registered to a reference MRI template (41–43) as previously described (37) and the following volumes-of-interest (VOIs) were selected for further analysis: basal ganglia, brainstem, cerebellum, cingulate cortex, orbito-frontal cortex, hippocampus, hypothalamus, insular cortex, midbrain, occipital cortex, parietal cortex, striatum, temporal cortex, thalamus, white matter and a VOI covering the whole brain. This registration procedure and region analysis have been previously described (37).

The registration of the images was performed as follows. First, the MRI of the animal was registered to the reference MRI using a 3 probability maps normalization (44). Then, (R)-[¹¹C]verapamil PET images were aligned to their corresponding MRI by rigid transformation, using a summation of all frames. For baseline scans, the (R)-[¹¹C]verapamil after-inhibition PET images were used as a reference, due to difficulties in the registration of the MRI with the baseline PET images (the latter showing lack of anatomical information in the image and limited brain radiotracer uptake).

2.4.3. Pharmacokinetic analysis

Several models were fitted to the VOI TACs, with the plasma corrected for polar radio-metabolites (metabolite-corrected plasma) or non-corrected plasma TACs and the whole-blood TACs as input function. The pharmacokinetic modeling was performed using PMOD v3.8 software.

First of all, the input functions from each animal were corrected for blood delay. The blood delay was calculated using the whole-brain TAC and then this value was fixed for the rest of the regions. The compartmental models evaluated were: the 1-Tissue Compartment Model (1-TCM), the 2-

Tissue Compartmental Model (2-TCM), and the Irreversible 2-TCM. All the models were assessed using either a fixed fractional blood volume (vB) (3, 4, 5, 6, or 7%) or using the vB as a fit parameter. Datasets with different scan durations (10-, 20-, 30-, 60- and 91-min) were analyzed in order to explore its effect on the model preference and the parameter's estimates. The compartmental model analyses were performed twice, once with the plasma corrected for polar radio-metabolites and then with the plasma non-corrected for radio-metabolites as input function. No boundary restrictions were applied for the fit of the parameters during the kinetic analysis.

Akaike Information Criterion (AIC) was used to choose the most optimal kinetic model for each scan duration and type of scan (baseline or after-inhibition). The standard errors (SE%) of the estimated parameters were also taken into consideration during model selection.

Several common kinetic parameters, i.e. the influx constant K_1 , the volume of distribution (V_T), and the efflux constant k_2 , were compared between baseline and after-inhibition scans to evaluate the effect of the inhibition and to select the most appropriate parameter to measure the P-gp function.

2.5. Parametric Images

Parametric images of K_1 , V_T , and k_2 at baseline and after-inhibition were calculated in one monkey (subject 2) for representation purposes. The metabolite-corrected plasma TAC was used as an input function for the basis function implementation of the 1-TCM, using PMOD v3.8 software.

2.6. Statistical Analysis

Inferential results are reported as Estimated Marginal Mean (EMM) $\pm$ standard error (SE) (mean $\pm$ SE) unless mentioned otherwise. Statistical analysis was performed using IBM SPSS Statistics version 23 (Armonk, NY, USA). Differences in kinetic parameter values and Akaike values among models, scan durations, and type of scans (baseline or after-inhibition) were assessed independently for each brain region by the Generalized Estimated Equation (GEE) model using an independent working correlation matrix (45,46). The GEE is recommended for the analysis of longitudinal data and it is also more efficient, achieving higher power with a small sample size (47). The relationship between the estimated parameters calculated with different models and different datasets was explored by linear regression analysis. Results were considered statistically significant at $p < 0.05$, without correction for multiple comparisons. The differences between baseline and after-inhibition are reported as a percentage, and calculated as follows: $100 \times (\text{After-inhibition} - \text{Baseline}) / \text{Baseline}$.

3. Results

3.1. Tracer production

Molar activities and radiochemical purities of (*R*)-[^{11}C]verapamil productions as well as the mass and radioactive dose injected in each animal are reported in table 2.

Table 2. Subject information and properties of (*R*)-[^{11}C]verapamil.

(<i>R</i>)-[^{11}C]verapamil					
Non-human primates	Scan	Body weight (Kg)	Injected dose (MBq)	Molar activity (GBq/ μmol)	Injected mass (nmol)
Subject 1	Baseline	8.2	893.6	118.2	7.6
Subject 1	After-inhibition	8.2	919.3	80.6	11.4
Subject 2	Baseline	6.1	968.8	20.5	47.4
Subject 2	After-inhibition	6.1	981.8	49.5	19.8
Subject 3	Baseline	7.2	1020.2	55.5	18.4
Subject 3	After-inhibition	6.6	941.1	85.3	11.0

3.2. Input function and metabolism of (*R*)-[^{11}C]verapamil.

A significant increase in the area under the curve of the whole-blood TACs was found after the administration of the P-gp inhibitor tariquidar (baseline = 2.03 ± 0.33 vs after-inhibition = 2.22 ± 0.35 , $p=0.041$). No statistically significant differences between baseline and after-inhibition scans were found in the uncorrected or metabolite-corrected plasma TACs (Figure 1). However, a statistically significant reduction of the % parent fraction of (*R*)-[^{11}C]verapamil was found after the P-gp inhibition ($75.25 \pm 1.37\%$, $p=0.019$) compared to baseline values ($79.82 \pm 1.26\%$). The K_e and $T_{1/2}$ of (*R*)-[^{11}C]verapamil did not significantly change after the P-gp inhibition.

Figure 1: (A) Metabolite-corrected plasma TACs of baseline (blue) and after-inhibition (green) scans (SUV). (B) Percentage of parent fraction at baseline (blue) and after-inhibition (green).

3.3. Plasma input models: Compartmental models

3.3.1. Blood Volume Fraction

After correcting the whole-blood and plasma TACs for blood delay, the fractional vB was either fixed to 3, 4, 5, 6, and 7% or used as fit parameter. According to the AIC, the best fits were obtained when the vB was used as fit parameter, which provided values ranging between 4-6%. Moreover, SE% of the estimated parameters were also lower when vB was fitted rather than fixed. Similar results were obtained when vB was fixed to 5%. Supplemental Figure 1 shows boxplots of AIC values and SE% K_1 values for the whole-brain using different scan duration and different models. Since fixing vB did not improve the fits, the following analyses have been performed using the vB as fit parameter.

3.3.2. Model selection for different scan durations

The 1-TCM and 2-TCM fits showed similar AIC values in short scans but in scans longer than 60-min, the lowest Akaike values were found using the 2-TCM. Figure 2 shows the AIC for the whole-brain for all tested scan durations and both scans (baseline and after-inhibition), obtained with 1-TCM, 2-TCM and Irreversible 2-TCM.

Figure 2: AIC in whole-brain using different models for all the scan durations at baseline (blue) and after-inhibition (green) scans. Mean values $\pm$ SE are plotted.

For the 60-min scan, even though the lowest AIC values were found using 2-TCM, the model showed very large SE% ($> 100\%$) in the estimation of k_2 , k_4 , and V_T . For instance, the mean SE% V_T of the whole-brain at baseline using 60-min scan duration was $14,898 \pm 12,158$. Using the 2-TCM, the SE% V_T values were in 35 cases (out of 96) higher than 100% and in 22 higher than 1000%. In the case of the 91-min scan duration, 2-TCM provided lower AIC values than 1-TCM, however, some regions showed unreliable values. In 13 cases (out of 96) the SE% V_T were higher than 100% and in 3 cases higher than 1000%. For these reasons, the 1-TCM (which showed lower SE%) was selected as the most robust model to analyze the data for all scan durations. Supplemental Figure 2 shows representative 1-TCM and 2-TCM fits for the 30- and 91-min scans of the whole brain.

3.3.3. Effect of scan duration

Based on our findings described above, the 1 TCM was used to fit the PET data for all tested scan durations. The evaluated scan durations did not affect significantly K_1 estimations (3-13%), at

least during the first 60 min of the scan. However, the estimation of k_2 decreased and the V_T increased with longer scan durations in both baseline and after-inhibition scans. In Figure 3, the effect of the scan duration on the kinetic parameters of the whole-brain in baseline and after-inhibition scans is shown.

Figure 3: K_1 (A), k_2 (B), and V_T (C) in whole-brain at baseline (blue) and after-inhibition (green), estimated from scans with different durations. Mean values $\pm$ SE are plotted.

V_T was most affected by the scan duration. In baseline scans, V_T increased by 61% from 2.16 ± 0.24 in a 10-min scan to 3.48 ± 0.38 ($p < 0.001$) in a 91-min scan. Similarly, in after-inhibition scans, the V_T increased by 40% from 7.88 ± 0.20 in 10-min to 10.99 ± 0.85 ($p < 0.001$) in a 91-min scan. The baseline V_T in the 60-min scan also increased by 40% ($p < 0.001$) and in after-inhibition scans by 29% ($p < 0.001$) compared to the values in the 10-min scan. The baseline k_2 decreased by 51% in a 91-min scan and by 36% in after-inhibition scans compared to a 10-min scan.

3.3.4. Effect of the P-gp inhibition

The v_B estimated by the model was not significantly different between baseline and after-inhibition scans for any of the scan durations, except for the 10-min scan duration where the estimated v_B significantly increased from 0.049 ± 0.001 at baseline to 0.052 ± 0.001 after P-gp inhibition ($p=0.018$).

In all scan durations, the parameter most affected by P-gp inhibition was V_T . In 91-min scans, the whole-brain V_T increased by 208% after P-gp inhibition meanwhile the K_1 increased by 159% in after-inhibition scans. The k_2 of the whole-brain decreased by 15%. In all scan durations, the V_T and K_1 significantly increased in all the brain regions after P-gp inhibition. Meanwhile, k_2 significantly decreased in all scan duration except for the 91-min scan. Table 3 shows the changes in V_T , K_1 , and k_2 after the P-gp inhibition in all the brain regions using 30- and 91-min scan duration.

Table 3: Changes in K_1 , V_T and k_2 after P-gp inhibition in brain regions using 30- and 91-min scan duration and 1-TCM. Mean values $\pm$ SE are listed, changes are shown as percentages.

% of changes between baseline and after-inhibition scan in 30-min scan							
Scan duration	Regions	$K_1 \pm SE$ (%)	p values	$V_T \pm SE$ (%)	p values	$k_2 \pm SE$ (%)	p values
30-min	Basal Ganglia	156 $\pm$ 45	<0.001	244 $\pm$ 38	<0.001	-25 $\pm$ 4	<0.001
	Brainstem	176 $\pm$ 46	<0.001	241 $\pm$ 33	<0.001	-19 $\pm$ 2	0.008
	Cerebellum	141 $\pm$ 37	<0.001	276 $\pm$ 42	<0.001	-35 $\pm$ 4	<0.001
	Cingulate Cortex	138 $\pm$ 32	<0.001	294 $\pm$ 41	<0.001	-40 $\pm$ 4	<0.001
	Orbito Frontal Cortex	93 $\pm$ 28	<0.001	190 $\pm$ 39	<0.001	-33 $\pm$ 3	<0.001
	Hippocampus	146 $\pm$ 35	<0.001	227 $\pm$ 32	<0.001	-25 $\pm$ 3	<0.001
	Hypothalamus	158 $\pm$ 44	<0.001	270 $\pm$ 39	<0.001	-30 $\pm$ 4	<0.001
	Insular Cortex	152 $\pm$ 38	<0.001	294 $\pm$ 36	<0.001	-35 $\pm$ 5	<0.001
	Midbrain	205 $\pm$ 57	<0.001	305 $\pm$ 49	<0.001	-25 $\pm$ 4	<0.001
	Occipital Cortex	130 $\pm$ 29	<0.001	268 $\pm$ 35	<0.001	-37 $\pm$ 4	<0.001
	Parietal Cortex	127 $\pm$ 31	<0.001	247 $\pm$ 34	<0.001	-34 $\pm$ 4	<0.001
	Striatum	168 $\pm$ 45	<0.001	267 $\pm$ 40	<0.001	-27 $\pm$ 3	<0.001
	Temporal Cortex	143 $\pm$ 41	<0.001	242 $\pm$ 39	<0.001	-28 $\pm$ 4	<0.001
	Thalamus	176 $\pm$ 41	<0.001	285 $\pm$ 33	<0.001	-28 $\pm$ 4	<0.001
	White Matter	145 $\pm$ 36	<0.001	268 $\pm$ 38	<0.001	-33 $\pm$ 4	<0.001
	Whole Brain	136 $\pm$ 34	<0.001	252 $\pm$ 37	<0.001	-33 $\pm$ 4	<0.001
% of changes between baseline and after-inhibition scan in 91-min scan							
91-min	Basal Ganglia	174 $\pm$ 46	<0.001	210 $\pm$ 42	<0.001	-11 $\pm$ 1	0.059
	Brainstem	199 $\pm$ 48	<0.001	199 $\pm$ 32	<0.001	1 $\pm$ 0	0.855
	Cerebellum	167 $\pm$ 41	<0.001	231 $\pm$ 43	<0.001	-18 $\pm$ 2	0.013
	Cingulate Cortex	166 $\pm$ 37	<0.001	237 $\pm$ 43	<0.001	-21 $\pm$ 2	0.041
	Orbito Frontal Cortex	111 $\pm$ 30	<0.001	153 $\pm$ 40	<0.001	-16 $\pm$ 2	0.049
	Hippocampus	162 $\pm$ 37	<0.001	198 $\pm$ 35	<0.001	-13 $\pm$ 2	0.160
	Hypothalamus	184 $\pm$ 46	<0.001	215 $\pm$ 43	<0.001	-9 $\pm$ 1	0.224
	Insular Cortex	177 $\pm$ 40	<0.001	246 $\pm$ 43	<0.001	-19 $\pm$ 2	0.004
	Midbrain	228 $\pm$ 62	<0.001	267 $\pm$ 52	<0.001	-11 $\pm$ 2	0.314
	Occipital Cortex	155 $\pm$ 34	<0.001	221 $\pm$ 34	<0.001	-20 $\pm$ 2	0.018
	Parietal Cortex	148 $\pm$ 34	<0.001	204 $\pm$ 36	<0.001	-18 $\pm$ 2	0.076

	Striatum	193±47	<0.001	220±41	<0.001	-8±-1	0.293
	Temporal Cortex	165±43	<0.001	199±41	<0.001	-10±-1	0.112
	Thalamus	200±43	<0.001	243±39	<0.001	-12±-1	0.222
	White Matter	170±39	<0.001	223±41	<0.001	-16±-2	0.094
	Whole-Brain	159±37	<0.001	208±40	<0.001	-15±-2	0.072

The magnitude of changes is dependent on scan duration since estimated values of V_T and k_2 are different in short and long scans. Figure 4 and Supplemental Figures 3 and 4 show the mean values of K_1 , V_T , and k_2 at baseline and after-inhibition.

Figure 4: Mean $\pm$ SE of K_1 for all brain regions at baseline and after-inhibition in scans with different durations.

For illustrative purposes, figure 5 shows V_T , K_1 , and k_2 parametric images of one subject (subject 2) at baseline and after-inhibition using 91-min scan and metabolite-corrected plasma as input function.

Figure 5: Parametric images of one subject using 91-min scan data at baseline (A) and after-Inhibition (B) representing K_1 ($\text{mL}/\text{cm}^3/\text{min}$, above), k_2 ($1/\text{min}$, center), and V_T (below).

3.3.5. Correlation K_1 - V_T

In 60- and 91-min scans, the K_1 and the V_T showed a good correlation at baseline ($R^2 = 0.74$, and $R^2 = 0.61$, $p < 0.01$) and after-inhibition scans ($R^2 = 0.70$ and $R^2 = 0.76$, $p < 0.01$; respectively). Figure 6 shows the correlation between K_1 and V_T in 60-min and 91-min scan for baseline and after-inhibition scans.

Figure 6: Correlation between K_1 and V_T obtained with 1-TCM using 60-min (A) or 91-min (B) data at baseline (blue) and after-inhibition (green).

3.4. Plasma non-corrected for radio-metabolites input models: compartmental models

The compartmental analyses were also performed using the plasma radioactivity non-corrected for radio-metabolites as input function for the 1-TCM in all scan durations. For most of the brain

regions analyzed, the SE% of K_1 , k_2 , and V_T were higher when using the plasma radioactivity non-corrected for radio-metabolites than with plasma corrected for polar radio-metabolites. In 91-min scan durations, the overall SE% V_T was 471% higher than the one obtained with the corrected-plasma. Moreover, V_T values obtained with non-corrected plasma were significantly lower than the ones obtained with plasma radioactivity corrected for polar radio-metabolites especially in after-inhibition scans (e.g. in 91-min after-inhibition scans V_T non-corrected plasma = 3.97 ± 0.27 vs. V_T corrected plasma = 10.87 ± 0.82 ; $p < 0.001$). The k_2 values obtained with non-corrected plasma were higher than in corrected-plasma in both baseline and after-inhibition scans and the differences were more pronounced at long scan duration (>60 -min). For instance, in a 91-min scan, the mean k_2 after-inhibition obtained with non-corrected plasma was 0.13 ± 0.014 1/min and with corrected plasma, it was 0.036 ± 0.001 1/min ($p < 0.001$).

Regarding the effect of the scan duration, the estimations of V_T decreased and k_2 increased with the scanning time when plasma non-corrected for radio-metabolites was used as input function whereas when plasma radioactivity was corrected for polar radio-metabolites, the estimations of V_T increased and the k_2 decreased (see Figure 7). Nevertheless, regarding the P-gp inhibition, similar results were obtained compared to the analysis with metabolite-corrected plasma. The administration of tariquidar significantly increased K_1 and V_T at all scan durations. However, the analysis did not find significant changes in k_2 after the P-gp inhibition. At 91-min scan duration, the K_1 increased from 0.20 ± 0.03 mL/cm³/min at baseline to 0.51 ± 0.06 mL/cm³/min after inhibition ($p < 0.001$) and the V_T from 1.71 ± 0.22 at baseline to 3.97 ± 0.27 after P-gp inhibition ($p < 0.001$).

Figure 7: k_2 (A) and V_T (B) at different scan durations at baseline and after-Inhibition using different input functions (plasma corrected for radio-metabolites or plasma non-corrected). Mean values $\pm$ SE are plotted.

4. Discussion

The aim of this study was to evaluate the kinetics of the P-gp tracer (*R*)-[^{11}C]verapamil in baseline conditions and after P-gp inhibition in non-human primates. Moreover, the study also evaluates the kinetics at different acquisition times (10- to 91-min) to assess the effect of the scan duration on the estimated parameters. Our results showed that the 1-TCM with fractional v_B as a fit parameter is the most suitable compartment model to fit the data at baseline and after-inhibition conditions for all the scan durations tested.

Model preferences seem not to be influenced by the scan duration. Although in the case of long scan durations (>60min) the lowest AIC were seen with the 2-TCM, large SE% (>100%) of the estimated parameters (V_T , k_2 , and k_4) were found. These large SE% did not provide reliable V_T values in several brain regions using 60- and 91-min scan durations. Several adjustments were applied to the model fit in an attempt to solve this problem. For instance, the use of 2-TCM while fixing the ratio K_1/k_2 to the whole brain grey matter value, as was suggested for the analysis of (*R*)-[^{11}C]verapamil in humans (19), or by imposing a lower boundary for the k_4 (0.001 1/min). However, none of these strategies improved the quality of the 2-TCM fit (results not reported). We have also tested a 2-input-compartmental model where we applied both a parent tracer and radio-metabolites plasma input curve at the same time in the model. This model has been already used in previous studies to account for the entrance of radio-metabolites of [^{11}C]verapamil in the brain (48). However, when using this model we found large uncertainties in the estimated kinetic parameters (K_1 , k_2 , and V_T) (with SE%>100%) in most of the brain regions analyzed (data not

shown). The 2-input model did not provide reliable and robust results for our data and was therefore not explored in our paper. Therefore, the 1-TCM was selected as the model of choice to analyze the (R)-[¹¹C]verapamil data in non-human primates for all tested scan durations and for both baseline and after-inhibition scans.

Although different kinetic analysis methods have been previously studied, a clear consensus regarding the optimal method has not yet been reached (14,19,23,49). In rats, the 2-TCM showed better fits than the 1-TCM for both baseline and after tariquidar administration scans. Thus, it was selected as the model of choice using either plasma corrected for polar radio-metabolites or plasma non-corrected for radio-metabolites (22,50). In humans, several approaches have been applied to fit the (R)-[¹¹C]verapamil data (14). In healthy subjects, the 1-TCM with the vB as a fit parameter and using a metabolite-corrected plasma input function showed an adequate fit. 2-TCM was also applied, however, the fit did not provide robust parameters (18). Next, (R)-[¹¹C]verapamil was also assessed after the administration of tariquidar (P-gp after-inhibition scans), in this case, a 2-TCM fit was preferred whereas the data from baseline scans (before the P-gp inhibition) was fitted with a 1-TCM using a plasma input function corrected for polar radio-metabolites (23). Muzi et al. used a 1-TCM to fit the 10-min scan duration data and a 2-TCM for the 45-min scan for both baseline and after P-gp inhibition scans, and corrected the plasma input function for polar and lipophilic radio-metabolites. In this study, Muzi et al. recommended the use of 10-min scan data to avoid the interference of radio-metabolites with the brain signal (49). Also, in humans, the use of constrained 2-TCM (i.e. fixing the ratio K_1/k_2 to the mean whole-brain grey matter value) was proposed, with plasma corrected for polar radio-metabolites as input function. The results of this analysis suggested that constrained 2-TCM leads to more reproducible results in healthy volunteers (19).

In our study, the administration of the P-gp inhibitor tariquidar caused a significant increase of V_T and K_1 in all the brain regions analyzed. V_T was the parameter most affected by P-gp inhibition. Tariquidar treatment caused an increase in V_T of 257% ($p < 0.001$) in the 10-min scan, of 230% ($p < 0.001$) in the 60-min scan, and an increase of 208% ($p < 0.001$) in 91-min scan compared to baseline. K_1 was also affected by P-gp inhibition. The changes in whole-brain K_1 between baseline and after-inhibition scans varied from 131% in 10-min scans to 159% in 91-min scans. Moreover, we also found a significant reduction in whole-brain k_2 at all the scan durations except for the 91-min scan. The k_2 was decreased by 34% in 10-min ($p < 0.001$) scans and by 15% in 91-min scans ($p = 0.072$). These results support the ability of (R)-[¹¹C]verapamil to detect changes in the P-gp function in non-human primates as has been already proven in other species.

Various studies have been done in humans under baseline and inhibition conditions [23,24]. Three hours after the administration of 2 mg/kg of tariquidar by intravenous infusion over 30-min caused

an increase of the V_T and K_1 by $24\pm15\%$ and $49\pm36\%$ respectively (23). Another study found that the administration of tariquidar as a continuous infusion during one hour before the PET scan and during the 60-min PET scan (total infusion time 2 hours and mean tariquidar dose 5.9 ± 1.0 mg/kg) increased V_T and K_1 values by $273\pm78\%$ and $259\pm74\%$ respectively, relative to baseline scans (24). Although the inhibition of the P-gp function caused also an increase in the K_1 and V_T values in humans, the values of the kinetic parameters and the effect size are different from the monkeys' values. However, since different doses of tariquidar, routes of administration, and duration of the perfusion were used in the monkeys, the human and monkey values cannot be directly compared. In rats, the P-gp inhibition also caused an increase in the K_1 and V_T , but similar discrepancies were observed (22,50).

Previous studies have shown that lipophilic radio-metabolites of (*R*)-[^{11}C]verapamil have similar kinetics as the parent tracer, thus most of the studies use the radiotracer concentration in plasma corrected for polar radio-metabolites as input function for the kinetic analysis (18,51). Our kinetic analysis was performed using the radiotracer concentration in plasma corrected for polar radio-metabolites and non-corrected for radio-metabolites as input function. When plasma non-corrected for radio-metabolites was used, large SE% ($>50\%$) in the parameter estimations were found. Therefore, corrections for polar radio-metabolites should be performed to avoid noisy data and low reliability of the estimated parameters. For long scan durations, V_T values calculated with plasma non-corrected for radio-metabolites as input were lower (40-60%) than with corrected plasma input, and k_2 values were 100-260% higher. These lower V_T values and the decrease in V_T estimations with increasing scan durations may be caused by the higher radioactivity concentration in the input function. Therefore, if the radioactivity in blood is not corrected by the amount of radio-metabolites, the model underestimates V_T values. When plasma-corrected for metabolites is used as input function the estimated V_T should not vary with the scan duration. However, since the plasma was only corrected by polar metabolites, it can be that lipophilic metabolites also accumulate in the brain which may cause the increase in the V_T estimations with longer scan durations. Concerning the tariquidar administration, the analysis using non-corrected plasma input showed a significant increase in V_T and K_1 after P-gp inhibition as was also observed when plasma corrected for polar radio-metabolites was used. In 91-min scans, V_T increased up to 132% ($p<0.001$) in after-inhibition scans relative to baseline and K_1 up to 150% ($p<0.001$). This similar effect of P-gp inhibition on the K_1 and V_T may allow avoiding the radio-metabolite analysis which is considered as a time-consuming and tedious process. However, if changes in the metabolism of (*R*)-[^{11}C]verapamil occur due to a disease condition or administration of treatments, the kinetic quantification can be incorrect. Therefore, the correction of plasma radioactivity for polar radio-metabolites, which have different kinetics than the parent fraction, should be performed, as was also concluded from human studies (18).

Our baseline V_T values using either metabolite-corrected or non-corrected plasma inputs were higher than those obtained with rats (22,50). However, K_1 values in rats and non-human primates at baseline conditions were similar. In our study, the mean whole-brain K_1 value at baseline was 0.15 ± 0.02 ml/cm³/min in 91-min scans using plasma corrected for radio-metabolites whereas in rats the K_1 values calculated with 2-TCM and plasma corrected for polar radio-metabolites were 0.16 ± 0.05 ml/cm³/min (50). Compared with human data, the K_1 and V_T values obtained in our study were also higher. The baseline K_1 values in humans were 0.034 ± 0.009 ml/cm³/min and increased to 0.049 ± 0.009 ml/cm³/min after P-gp inhibition. The V_T values in 40 min scans were 0.65 ± 0.13 at baseline and 0.80 ± 0.07 after inhibition, as calculated with 2-TCM (23). Comparing with our 30-min scan duration data, baseline K_1 values in non-human primates were 429% higher relative to humans and the baseline V_T values were 288% higher. These different values could be caused by species differences in the expression of P-gp at the BBB (52,53).

Kinetic evaluation of another strong P-gp substrate tracer, [¹¹C]-N-desmethyl-loperamide ([¹¹C]dLop), in non-human primates before and after administration of the P-gp inhibitor cyclosporine A (54), showed similar increases of brain uptake after P-gp inhibition as were observed in our study. The evaluation of [¹¹C]dLop did not use compartmental models and, therefore, the brain uptake was not calculated using the V_T but, using the ratio between the area under the curve (AUC) from the brain and the AUC from the blood ($AUCR = AUC_{\text{brain}}/AUC_{\text{blood}}$) (54). In this study, the AUCR after the administration of the P-gp inhibitor (15 mg/kg/h i.v.) was enhanced to 10.8 ± 3.6 (54), which is similar to our after-inhibition V_T value of the whole-brain of 10.87 ± 0.82 .

With regards to the tracer kinetics in plasma, it is known that (R)-[¹¹C]verapamil suffers from extensive *in vivo* metabolism, both in small animals and humans. In the present study in non-human primates, the percentage of parent tracer at 30-min after the injection was 57% in baseline scans and 45% in after-inhibition scans, after correction for polar radio-metabolites. Regarding the differences in parent tracer between baseline and after-inhibition scans, our analysis found a significant reduction (6%) in the percentage of parent (R)-[¹¹C]verapamil in after-inhibition scans compared to baseline. In humans, the percentage of parent tracer is around 45% at 1 hour after the injection (18). In rats, at 30-min after tracer injection, the fraction of parent (R)-[¹¹C]verapamil in plasma was 47%, and after 1 hour, it was 27% (55). Previous radio-metabolite studies with non-human primates, performed by HPLC, have already reported a strong metabolism of (R)-[¹¹C]verapamil with around 80% of plasma radioactivity associated with radio-metabolites (polar and lipophilic radio-metabolites) at 30-min after the injection (35). Therefore, the rate of metabolism of (R)-[¹¹C]verapamil appears to be rapid and similar in different species.

Short PET scan durations are more convenient for the subjects and also to simplify the kinetic evaluation by reducing the impact of tracer metabolism. In our study, K_1 values slightly decreased (3-13%) with increasing scan duration but remained relatively constant regardless of scan duration applied. V_T was most affected by the scan duration. In the 91-min scan, the V_T increased up to 61% ($p < 0.001$) in baseline and 40% ($p < 0.001$) in after-inhibition scans compared to values obtained with 10-min scan duration. We also observed a decrease in k_2 values (by 51% in baseline and 36% after-inhibition) with increasing scan durations. This coupled increase in the V_T and decrease in the k_2 estimations might be caused by the accumulation of radio-metabolites in the brain. As was found for rats and humans, radio-metabolites formed by the metabolism of (*R*)-[^{11}C]verapamil can cross the BBB and accumulate inside the brain. This fact may complicate the kinetic analysis of the tracer since it is not possible to differentiate the origin of the radioactive signal (radio-metabolites or parent tracer). Thus, short PET acquisition times have been proposed for the analysis of (*R*)-[^{11}C]verapamil (14,49,55). Since the K_1 is less affected by the scan duration than the V_T , we recommend the use of K_1 calculated using 1-TCM and short PET scan durations to measure the P-gp function at the BBB of non-human primates, preferably accomplished with quantitative perfusion PET studies allowing to differentiate between P-gp function and perfusion changes.

A potential limitation of our study is the small sample size, though the longitudinal design may reduce the subject variability and the outcomes show strong and similar inhibitory effects in all the subjects which emphasize the robustness of our results. It is also unfortunate that the study did not analyze lipophilic radio-metabolites in the blood samples. However, this analysis would require larger volumes of blood during blood sampling which could be harmful to the animals and lead to errors in the pharmacokinetic analysis. An additional limitation is the position of the animals in the PET scan. In our study, the animals were sitting in the PET scan whereas in conventional PET scanners the subjects used to be in a supine position. This fact may change the biologic function of the animals and thus alter the kinetics of the tracer. Thereby, the kinetic comparison with other PET studies must be interpreted with caution. However, this position enables the performance of the PET scans in conscious unanesthetized monkeys, thus anesthetics will not interfere in the distribution of the tracer (56). Moreover, our study did not correct for partial volume effect, thus the radioactivity of some small brain regions may be affected by spill-over from regions with high tracer uptake.

This study has provided more insight into the pharmacokinetic parameters of (*R*)-[^{11}C]verapamil in non-human primates allowing the comparison with other species. Moreover, these results enable the head-to-head comparison of the properties of a novel P-gp PET tracer, such as [^{18}F]MC225 (37) or [^{11}C]metoclopramide (31), with the P-gp tracer, (*R*)-[^{11}C]verapamil, in non-human primates, a species larger than rodents and physiologically more similar to humans (57).

5. Conclusion

Our results suggest that the 1-TCM is the model of choice to analyze PET data of (*R*)-[¹¹C]verapamil in non-human primates. The model preference is not affected by scan duration and a similar approach should be used in baseline scans when the P-gp function is fully functional and in scans after P-gp inhibition. V_T and K_1 values obtained in non-human primates were different from humans and rats with the same radiotracer, which highlights P-gp expression differences among species. However, the rate of (*R*)-[¹¹C]verapamil metabolism seems similar in all the species examined. V_T was the parameter most affected by the challenge (P-gp inhibition). However, since estimated V_T change with scan duration and thus cannot be reliably determined, we recommend the use of K_1 calculated with 1-TCM as a suitable parameter to measure the P-gp function at the BBB of non-human primates. Moreover, short scan durations are recommended to avoid quantification being affected by the presence of radio-metabolites in the brain.

6. Supporting Information

Supplemental Figure 1: Box plot of whole-brain AIC values (A) and SE%K₁ values (B) using different vBs at different models, scan durations, and type of scan (baseline and after-inhibition).

Supplemental Figure 27: Representative 1-TCM (purple) and 2-TCM (blue) of one subject using 30-min (A) and 91-min scan duration (B) at baseline (squares) and after-inhibition (circles).

Supplemental Figure 3: Mean $\pm$ SE of k_2 of all the brain regions at baseline and after-inhibition scans using different scan durations.

Supplemental Figure 4: Mean $\pm$ SE of VT of all the brain regions at baseline and after-inhibition scans using different scan durations.

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Chapter 6

Head to head comparison of (*R*)-[¹¹C]verapamil and [¹⁸F]MC225 in non-human primates, tracers for measuring P-glycoprotein function

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Abstract

Purpose: P-glycoprotein (P-gp) function is altered in several brain disorders; thus, it is of interest to monitor the P-gp function in vivo using PET. (*R*)-[^{11}C]verapamil is considered as the gold standard tracer to measure the P-gp function, however, it presents some drawbacks that limit its use. New P-gp tracers have been developed with improved properties, such as [^{18}F]MC225. This study compares the characteristics of (*R*)-[^{11}C]verapamil and [^{18}F]MC225 in the same subjects.

Methods: Three non-human primates underwent 4 PET scans: 2 with (*R*)-[^{11}C]verapamil and 2 with [^{18}F]MC225, at baseline and after P-gp inhibition. The 30-min PET data were analyzed using 1-Tissue Compartment Model (1-TCM) and metabolite-corrected-plasma as input function. Tracer kinetic parameters at baseline and after-inhibition were compared. Regional differences and simplified methods to quantify the P-gp function were also assessed.

Results: At baseline, [^{18}F]MC225 V_T values were higher and k_2 values were lower than those of (*R*)-[^{11}C]verapamil, whereas K_1 values were not significantly different. After-inhibition, V_T values of the 2 tracers were similar, however, (*R*)-[^{11}C]verapamil K_1 and k_2 values were higher than those of [^{18}F]MC225. Significant regional differences between tracers were found at baseline, which disappeared after inhibition. The positive slope of the SUV-TAC was positively correlated to the K_1 and V_T of both tracers.

Conclusion: [^{18}F]MC225 and (*R*)-[^{11}C]verapamil show comparable sensitivity to measure the P-gp function in non-human primates. Moreover, this study highlights the 30-min V_T as the best parameter to measure decreases in the P-gp function with both tracers. [^{18}F]MC225 may become the first radiofluorinated tracer able to measure decreases and increases in the P-gp function due to its higher baseline V_T .

Keywords: Brain imaging, Central Nervous System, Efflux Transporter, Regional Differences

1. Introduction

ATP binding cassette (ABC) transport proteins located at cerebral endothelial cells in the blood-brain barrier (BBB) pump out a wide variety of compounds from the brain to the blood (1,2), contributing to the maintenance of cerebral homeostasis and the protection of the central nervous system (CNS) (3,4). These transporters use ATP hydrolysis to transport substrates from the intracellular to the extracellular compartment (5,6). P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP), and Multidrug Resistance-Associated Protein 1 (MRP-1) are the most studied ABC transporters, because of their clinical relevance (7,8).

The P-gp transporter can recognize a large number of structurally diverse exogenous compounds such as anti-cancer, anti-epileptic, and antidepressant drugs, and also endogenous compounds. Inflammatory responses, stress, therapeutic drugs, and diet can modify the expression and/or function of the P-gp transporter (9,10). An increase in the P-gp function has been related to decreases in drug efficiency (drug resistance) (1,11). This phenomenon is especially important in the treatment of brain tumors (12), intractable epilepsy (13), psychiatric diseases (1), and infectious diseases (14). On the other hand, a decline in the P-gp activity is associated with an increased concentration of neurotoxic compounds inside the CNS, which may be related to the onset of several neurodegenerative diseases (15,16) or may cause neurological problems (17,18). Vogelgesang et al. demonstrated that the β -amyloid deposition inside the brain, which is the pathological hallmark of Alzheimer's disease (19,20), is inversely correlated with the P-gp expression in endothelial cells of cerebral blood vessels (21).

Assessment of the P-gp function in vivo may help to diagnose several neurodegenerative diseases and may predict the efficacy of CNS treatments. PET imaging has already been used to study the P-gp function at the BBB in humans (22–26). Nowadays, (R)-[¹¹C]verapamil and [¹¹C]-N-desmethyl-loperamide are considered as “gold standard” tracers for imaging the P-gp function, being the most extensively used in preclinical and clinical research (24,25,27). However, these tracers have been identified as strong P-gp substrates (22,28,29), i.e. the tracers are quickly transported from the brain to the blood. This results in a low tracer concentration inside the brain (25), precluding their use in the assessment of P-gp upregulation, which might occur in treatment-resistant depression (30) and patients with intractable epilepsy (13).

For these reasons, many efforts have been made to develop new P-gp tracers with improved pharmacokinetic properties and lower affinity to the P-gp transporter (25). [¹¹C]metoclopramide (31), [¹¹C]emopamil (32), [¹¹C]phenytoin (26), and [¹⁸F]MC225 (33) were identified as weak substrates of the P-gp transporter, showing higher tracer uptake in the brain than (R)-

[^{11}C]verapamil at baseline conditions when P-gp is functioning adequately. [^{18}F]MC225 was selected as the most promising fluorine-18 labeled tracer for in vivo measurement of P-gp function (34). Recently, the kinetic properties of [^{18}F]MC225 were evaluated, and the results confirmed the ability of this tracer to measure changes in the P-gp function of rats (33) and non-human primates (35).

The present study is the first direct comparison of the characteristics of the weak P-gp substrate [^{18}F]MC225 and the strong P-gp substrate (*R*)-[^{11}C]verapamil in non-human primates. To this aim, the function of the P-gp transporter was explored in three rhesus monkeys (*Macaca mulatta*) under normal conditions as well as after the administration of the P-gp inhibitor, tariquidar. Based on previous publications that analyzed the pharmacokinetics of [^{18}F]MC225 and (*R*)-[^{11}C]verapamil in non-human primates (35,36), the 1-Tissue Compartment Model (1-TCM) was fitted to the data of both tracers. Kinetic parameters such as the influx constant K_1 , the volume of distribution (V_T), and the efflux constant k_2 were compared between the tracers at baseline and after-inhibition. Regional differences between tracers were also analyzed, and simplified methods to quantify the P-gp function were assessed.

2. Experimental Section

2.1 Tracer Production

The radiosynthesis and quality control of (*R*)-[^{11}C]verapamil and [^{18}F]MC225 were performed as described previously (35–37).

2.2 Animals Experiments and Study Plan

All animal procedures were carried out in accordance with the recommendations of the National Institutes of Health (NIH), the guidelines of the Ethics Committee of the Central Research Laboratory, Hamamatsu Photonics (approval HPK-2016-07A), and the Institutional Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology (approval 16067).

Three healthy male rhesus monkeys (*Macaca mulatta*; Hamri Co. Ltd., Ibaraki, Japan) were individually housed in a controlled room with a temperature of $24 \pm 4^\circ\text{C}$, a humidity of $50 \pm 20\%$, and under a 14-hour light/10-hour dark cycle. Each monkey underwent a single MRI and 4 PET scans (Figure 1) with (*R*)-[^{11}C]verapamil and [^{18}F]MC225. First, PET scans were performed using (*R*)-[^{11}C]verapamil at baseline and after P-gp inhibition with tariquidar (after-inhibition scan). The interval between these scans was two hours in two animals, and two months in the third animal (due to technical problems). Approximately one month later, PET scans with [^{18}F]MC225

were acquired, with an interval of one month between the baseline and after-inhibition scan. Inhibition of the P-gp function was achieved by intravenous administration of the P-gp inhibitor tariquidar (MedChemExpress, New Jersey, USA) at a dose of 8 mg/kg body weight, 15 min before the PET scan.

Figure 1: Schematic of PET protocol. First, each animal underwent an MRI scan (to acquire anatomic information) and subsequently, the PET scans were made, first using (R)-[¹¹C]verapamil and later [¹⁸F]MC225.

2.3 Imaging Experiments

First, a T1-weighted brain MRI scan of each subject was acquired (Signa Excite HDTx 3.0T scanner, GE Healthcare). Afterward, animals underwent a 60-min-transmission scan using a rotating ⁶⁸Ge/⁶⁸Ga rod source followed by a 91-min-dynamic emission PET scan (SHR-38000, Hamamatsu Photonics) with arterial blood sampling. Animals were injected with (R)-[¹¹C]verapamil (954.2 ± 45.6 MBq, with $99.3 \pm 0.2\%$ radiochemical purity and a molar activity (MA) higher than 20 GBq/ μ mol), or [¹⁸F]MC225 (684 ± 64 MBq, with a purity of $97.6 \pm 1.0\%$ and a MA higher than 36 GBq/ μ mol), as a single bolus (over 30 seconds) via the saphenous vein, at the start of the emission scan (for more information (35,36)).

2.4 Arterial Blood Sampling and Analysis

Immediately after administration of the tracer and during the PET scan, blood samples (0.5 ml) were drawn from a cannula placed in the posterior tibial artery. Plasma and blood were separated according to the protocol described elsewhere (35). Parent fraction and radioactive polar metabolites of (R)-[¹¹C]verapamil and [¹⁸F]MC225 in plasma were determined as previously described (35,36). Standardized Uptake Values (SUV) time-activity curves (TAC) were calculated as previously described (35,36).

2.5 Image Preprocessing

PET data were corrected for attenuation, using the transmission scan. Reconstruction of PET images, image registration, and analysis were performed as previously described (35,36). Briefly,

MRI images were individually co-registered to an anatomical atlas and to the PET images. Several volumes of interest (VOIs) were extracted from the MRI data co-registered to the brain atlas (38).

2.6 PET Imaging Analysis

Simplified quantification methods: Standardized Uptake Values

For each PET scan, TACs were generated for the brain regions and corrected for the body weight and the dose of radioactivity injected to obtain SUV-TACs. Maximum values of each SUV-TAC of the 30 min scan were also extracted for both tracers from baseline and after-inhibition scans.

Pharmacokinetic Modeling

The metabolite-corrected plasma and the whole-blood TACs were used as an input function to perform pharmacokinetic modeling using PMOD (PMOD Technologies, v3.8, Zürich, Switzerland).

Previous publications have already selected the 1-TCM to fit the (R)-[¹¹C]verapamil and [¹⁸F]MC225 data of baseline and after-inhibition scans using short scan durations (<30 min) (35,36). Longer scan durations are more affected by the metabolism of the tracers since radio-metabolites can interfere with the brain signal and lead to erroneous measurements. Thus, this study focuses on the 30-min scan duration and the 1-TCM was fitted to regional TACs of both tracers.

K_1 , V_T , and k_2 values from each brain region were compared between tracers at baseline and in after-inhibition scans. The effect size caused by P-gp inhibition was also compared between tracers. Moreover, a global scaling of the K_1 , V_T , and k_2 values was applied to study the regional differences relative to the whole-brain for both tracers and scans. To this aim, the K_1 , V_T , and k_2 values of each region were normalized to the values of the whole-brain (e.g., normalized $K_{1,i} = \text{VOI}_i * K_{1,i} / \text{VOI}_{wb} * K_{1,wb}$ being VOI_i the volume of the region i , $K_{1,i}$ the value of the region i , VOI_{wb} the volume of the whole-brain and $K_{1,wb}$ the value of the whole-brain). Relative regional differences between the tracers were compared in baseline and in after-inhibition scans. Regional changes caused by the P-gp inhibition were also related to the changes in the whole-brain and were compared between tracers.

The negative slope (also called the elimination constant (K_{el})) calculated using the SUV-TAC of the brain regions has been proposed as a simplified method to measure the P-gp function (39,40). We also calculated by linear regression the positive slope of regional SUV-TACs using the first 75 seconds of the scan. The slopes were compared to the K_1 and V_T values of both tracers.

2.8 Parametric Images

An average parametric image representing K_1 , V_T , and k_2 values of the brain was calculated for both tracers and scans (baseline and after-inhibition) using 30-min scan duration. The metabolite-corrected plasma TAC was used as an input function for 1-TCM basis functions, using PMOD. Moreover, parametric images showing the changes in K_1 , V_T , and k_2 due to P-gp inhibition relative to baseline were also calculated using MATLAB (The MathWorks, Inc). These images highlight the regions most affected by the P-gp inhibition for each tracer.

2.9 Statistical Analysis

Results are presented as mean±standard error (SE) unless mentioned otherwise. Statistical analysis was performed using IBM SPSS Statistics version 23 (Armonk, NY, USA). Differences between tracers and scans were assessed for each brain region by generalized estimated equation (GEE) with independent matrix (41,42). Results were considered statistically significant at $p < 0.05$, without correction for multiple comparisons. The differences between baseline and after-inhibition scans were calculated as follows: $(V_T \text{ after-inhibition} - V_T \text{ Baseline}) / V_T \text{ Baseline}$. The differences between tracers were calculated as: $(V_T [^{18}\text{F}]MC225 - V_T (R)-[^{11}\text{C}]verapamil) / V_T (R)-[^{11}\text{C}]verapamil$, taking (R)-[¹¹C]verapamil as the reference. Both values are expressed as percentages.

3. Results

3.1. Whole-brain SUV-TACs

At baseline, [¹⁸F]MC225 maximum SUV values were 64% ($p < 0.001$) higher than those of (R)-[¹¹C]verapamil. [¹⁸F]MC225 SUV values continue to slightly increase after the initial sharp rise to a final value of 1.19 ± 0.03 . In (R)-[¹¹C]verapamil, the initial sharp rise led to a maximum value of 0.73 ± 0.03 , which was reached at 48 ± 6 seconds. Tariquidar significantly increased the SUV-TACs for the whole-brain of both tracers (Figure 2). The maximum values increased by 82% ($\text{SUV} = 2.17 \pm 0.10$) for [¹⁸F]MC225 and by 184% ($\text{SUV} = 2.07 \pm 0.13$) for (R)-[¹¹C]verapamil. Moreover, the shape of the SUV-TACs completely changed after P-gp inhibition. In the case of [¹⁸F]MC225, a steeper increase was observed compared to baseline scans. In the case of (R)-[¹¹C]verapamil, P-gp inhibition caused a delay in the peak value (from 48 ± 6 seconds to 17 ± 6 minutes). The maximum SUV values of the two tracers were not significantly different in after-inhibition scans ($p = 0.155$).

Figure 2: SUV-TAC of the whole-brain for both tracers at baseline (left) and after P-gp-inhibition (right).

3.2 Brain Kinetics of [^{18}F]MC225 and (*R*)-[^{11}C]verapamil.

3.2.1. Global changes in K_1 , k_2 , and V_T

Figure 3 shows the average parametric maps of K_1 , k_2 , and V_T of both tracers. The figure shows how the V_T and K_1 values of both tracers increased after P-gp inhibition and (*R*)-[^{11}C]verapamil k_2 values decreased. At baseline, whole-brain V_T values were 140% higher for [^{18}F]MC225 (6.05 ± 0.45) than for (*R*)-[^{11}C]verapamil (2.52 ± 0.32) ($p < 0.001$), while k_2 values were 59% lower for [^{18}F]MC225 (0.03 ± 0.01) than for (*R*)-[^{11}C]verapamil (0.07 ± 0.001) ($p < 0.001$). By contrast, whole-brain K_1 values of both tracers at baseline were not significantly different ($p = 0.718$) (K_1 [^{18}F]MC225 = 0.17 ± 0.01 and K_1 (*R*)-[^{11}C]verapamil = 0.18 ± 0.03). After P-gp inhibition, the whole-brain K_1 and the k_2 of [^{18}F]MC225 were 49% and 37% lower than those of (*R*)-[^{11}C]verapamil (K_1 [^{18}F]MC225 = 0.24 ± 0.01 and K_1 (*R*)-[^{11}C]verapamil = 0.42 ± 0.05 ; $p < 0.001$) (k_2 [^{18}F]MC225 = 0.03 ± 0.001 and k_2 (*R*)-[^{11}C]verapamil = 0.05 ± 0.004 ; $p < 0.001$). Meanwhile, the whole-brain V_T of [^{18}F]MC225 (8.04 ± 0.24) was not significantly different from the V_T of (*R*)-[^{11}C]verapamil (8.87 ± 0.20) ($p = 0.058$).

Comparisons between V_T , K_1 , and k_2 values obtained from baseline and after inhibition scans show that the effect size of the P-gp inhibition was larger in the case of (*R*)-[^{11}C]verapamil. K_1 increased up to 135.8% for (*R*)-[^{11}C]verapamil ($p < 0.001$) after P-gp inhibition and up to 39.1%

for [¹⁸F]MC225 ($p < 0.001$). V_T increased up to 252% for (R)-[¹¹C]verapamil ($p < 0.001$) and to 33% for [¹⁸F]MC225 ($p = 0.001$). Values of k_2 decreased by 32.9% for (R)-[¹¹C]verapamil ($p < 0.001$) and did not significantly change for [¹⁸F]MC225 after P-gp inhibition. Supplemental Figure 2 illustrates regional differences of K_1 , k_2 , and V_T for both tracers.

Figure 3: Parametric maps of K_1 (above), k_2 (middle), and V_T (below) of both tracers at baseline and after-inhibition (left). Percentage of change due to the P-gp inhibition in K_1 (above), k_2 (middle), and V_T (below) for [¹⁸F]MC225 and (R)-[¹¹C]verapamil (right).

3.2.2. Regional changes in K_1 , k_2 , and V_T

As expected from Figure 3, significant differences between brain regions were found for K_1 , k_2 , and V_T values. The spatial distribution of K_1 , k_2 , and V_T values and, in particular, the comparison between tracers and the impact of P-gp inhibition was carried out using the global changes as a confounder. Thus, K_1 , k_2 , and V_T values normalized to the whole-brain region of both tracers at both scans are represented in Figure 4.

Regarding the baseline values of (R) - $[^{11}\text{C}]$ verapamil, regions such as the occipital and orbitofrontal cortex provided a higher relative contribution to the whole-brain K_1 , whereas, for the whole-brain V_T the largest contributions were from the orbitofrontal and temporal cortex. In the case of whole-brain k_2 values, occipital cortex and cerebellum provided the highest relative contribution. Regarding the baseline K_1 and V_T values of $[^{18}\text{F}]$ MC225, the regions with the highest contribution were occipital cortex and cerebellum, while occipital cortex and midbrain were the main contributors to the whole-brain baseline k_2 values. These different relative contributions lead to regional differences between the tracers at baseline. For instance, in the case of $[^{18}\text{F}]$ MC225, the midbrain and orbitofrontal cortex K_1 values were 15% higher and 14% lower than those of (R) - $[^{11}\text{C}]$ verapamil, respectively. Similarly, V_T values of $[^{18}\text{F}]$ MC225 were 38% higher in the cerebellum and 20% lower in the orbitofrontal cortex than those of (R) - $[^{11}\text{C}]$ verapamil. Also, k_2 values of $[^{18}\text{F}]$ MC225 were 21% lower than those of (R) - $[^{11}\text{C}]$ verapamil in the cerebellum and 19% higher in the basal ganglia.

In after-inhibition scans, midbrain and occipital cortex provided the highest contribution to the whole-brain K_1 of (R) - $[^{11}\text{C}]$ verapamil and occipital cortex and cerebellum for $[^{18}\text{F}]$ MC225. Insular and cingulate cortex were the regions with the highest contribution to the whole-brain V_T of both tracers. Regarding whole-brain k_2 after-inhibition values, cerebellum and occipital cortex provided the highest contribution for (R) - $[^{11}\text{C}]$ verapamil whereas brainstem and cerebellum provided the highest contribution for $[^{18}\text{F}]$ MC225. Although different relative contributions were also observed in after-inhibition scans, the regional differences between the tracers disappeared (regional differences between tracers were lower than 9%).

Due to the differences in the $[^{18}\text{F}]$ MC225 K_1 , V_T , and k_2 values in various brain regions compared to the values of (R) - $[^{11}\text{C}]$ verapamil, the tracers showed also different changes of uptake due to the P-gp inhibition as can be observed in Figure 5 and Supplemental Table 1.

Figure 4: Regional differences in K_1 , k_2 , and V_T values relative to the whole-brain region at baseline and after-inhibition with both tracers.

Figure 5: Relative changes of K_1 , k_2 , and V_t due to P-gp inhibition in all brain regions for the two tracers.

3.3. Correlations: SUV-Kinetic parameters

Since the SUV-TAC did not show any negative slope during the 30-min of the scan duration, the K_{eb} could not be calculated and correlated to the pharmacokinetic parameters. On the other hand, the positive slope of the SUV-TACs was positively correlated with the K_1 (R^2 (R)-[¹¹C]verapamil=0.63 ($p<0.001$) and R^2 [¹⁸F]MC225=0.61 ($p<0.001$)) and V_T (R^2 (R)-[¹¹C]verapamil=0.81 ($p<0.001$) and R^2 [¹⁸F]MC225=0.64 ($p<0.001$)) values of both tracers (see Supplemental Figure 3).

4. Discussion

The present study compares the pharmacokinetic parameters of the novel P-gp PET tracer [¹⁸F]MC225 and the gold standard P-gp tracer (R)-[¹¹C]verapamil in non-human primates, under normal conditions as well as after P-gp inhibition. The data of both tracers were assessed using a 30-min scan duration, to avoid the presence of radio-metabolites. The 1-TCM was the model fitted to the data of both tracers, following the recommendations in previous publications (35,36). The kinetic parameters (K_1 , k_2 , and V_T) of the two tracers in baseline and after-inhibition scans were compared.

We found that in baseline scans, the V_T of [¹⁸F]MC225 was significantly higher than the V_T of (R)-[¹¹C]verapamil. This finding was expected because (R)-[¹¹C]verapamil is considered as a strong substrate of P-gp and, consequently, is quickly transported from brain to blood, resulting in a low brain uptake. Meanwhile, [¹⁸F]MC225 is known as a weak substrate of P-gp transporter, thus the baseline concentration of [¹⁸F]MC225 inside the brain is higher. This finding is in line with previous studies performed in mice where the whole-brain SUV values of [¹⁸F]MC225 were higher than those of (R)-[¹¹C]verapamil (34). Nevertheless, our study showed that these differences in V_T do not arise from differences in K_1 , since K_1 values were not significantly different between tracers at baseline. They are caused by differences in k_2 values, which were significantly lower for [¹⁸F]MC225 than for (R)-[¹¹C]verapamil.

Administration of the P-gp inhibitor tariquidar increased the V_T and K_1 values of both tracers in all brain regions, while the efflux constant k_2 was decreased for (R)-[¹¹C]verapamil and remained unchanged for [¹⁸F]MC225. Since in 1-TCM, $V_T = K_1/k_2$, the rise in V_T after P-gp inhibition in [¹⁸F]MC225 scans is mainly caused by the increase in the K_1 . Based on the Fick principle and Renkin-Crone model (43,44), K_1 depends on the blood flow (F) and the extraction fraction (E_u) of the tracer ($K_1 = F * E_u$). If K_1 changes were caused by changes in the blood flow, then k_2 values would also be increased ($k_2 = K_1/V_T$). Since this was not the case, K_1 changes seem to be caused

by an increase in the extraction fraction of the tracer which can be related to increased permeability of the capillaries due to the P-gp inhibition. On the other hand, the increase in the (R) - $[^{11}\text{C}]$ verapamil V_T is caused by the increase in K_1 and decrease in k_2 values. The changes in k_2 are negligible (-32%) compared to changes observed in V_T (+252%) and K_1 (+136%). Thus, the increase in V_T is mainly related to the increase in K_1 which is caused by the P-gp inhibition. For this reason, previous publications support the use of K_1 as the best parameter to measure the P-gp function at the BBB, confirming the ability of both tracers to detect decreases in the P-gp function at the BBB of non-human primates (35,36).

Even though both tracers can detect decreases in the P-gp function, the low V_T values of (R) - $[^{11}\text{C}]$ verapamil at baseline could hamper the quantification of increases in the P-gp function, which would be associated with a further decrease in tracer V_T . $[^{18}\text{F}]\text{MC225}$ does not show this limitation since it has a higher baseline V_T than (R) - $[^{11}\text{C}]$ verapamil. Moreover, the ability of $[^{18}\text{F}]\text{MC225}$ to measure increases in the P-gp function has been confirmed, since the administration of a P-gp inducer to healthy rats, decreased the V_T and K_1 values of $[^{18}\text{F}]\text{MC225}$ compared to controls (45). Since K_1 values at baseline were similar for both tracers, it could be expected that the (R) - $[^{11}\text{C}]$ verapamil K_1 may be able to reflect increases in the P-gp function. However, most studies have failed to measure increases in the P-gp function using this parameter. For instance, (R) - $[^{11}\text{C}]$ verapamil scans in patients with chronic schizophrenia and major depression did not show significant differences in K_1 values compared to controls (46,47). Also, (R) - $[^{11}\text{C}]$ verapamil was used to detect increased P-gp function in epileptic patients, but the authors did not find any significant differences in the K_1 and V_T between epileptic brain tissue and its contralateral healthy tissue (48).

The present study also found that K_1 and k_2 values of (R) - $[^{11}\text{C}]$ verapamil were significantly higher than those of $[^{18}\text{F}]\text{MC225}$, whereas V_T values of both tracers after P-gp inhibition were not significantly different. Since the animals were injected with the same dose of tariquidar (8 mg/Kg) and the same injection protocol was used, similar after-inhibition kinetic values were expected for both tracers, in particular, for K_1 and V_T . It was expected that after the P-gp inhibition, both tracers could enter the brain regions reaching similar values, as was the case for V_T . Thus, one could argue that the K_1 of (R) - $[^{11}\text{C}]$ verapamil may not be an adequate parameter to measure the P-gp function. The higher K_1 values of (R) - $[^{11}\text{C}]$ verapamil after P-gp inhibition together with the lack of sensitivity of (R) - $[^{11}\text{C}]$ verapamil K_1 values to detect increases in P-gp function, may suggest that K_1 of (R) - $[^{11}\text{C}]$ verapamil is affected by other non-specific factors. This conclusion is supported by in vitro studies which found that $[^{18}\text{F}]\text{MC225}$ is more specific for P-gp than (R) - $[^{11}\text{C}]$ verapamil (34).

Our results also confirmed the presence of significant regional differences in the K_1 , V_T , and k_2 distributions for both tracers at baseline. Overall, the highest baseline V_T and K_1 values for both tracers were found in cortical regions such as occipital and orbitofrontal cortex, and in the case of [¹⁸F]MC225 higher V_T and K_1 values were also found in the cerebellum. These findings agree with previous publications where the highest uptake at baseline for both tracers in rats was found in frontal cortex and cerebellum (33,49). Since at baseline the P-gp function is working adequately, this may suggest that cerebellum and cortical regions display a lower P-gp function compared to other brain regions. However, after the P-gp inhibition, the regional differences between tracers were reduced, indicating that some regions were more affected by P-gp inhibition than others. In (R)-[¹¹C]verapamil the most affected region was the midbrain whereas in [¹⁸F]MC225, it was the striatum. For both tracers, the regions less affected by P-gp inhibition were the cerebellum and orbitofrontal cortex. These results also suggest that subcortical areas may have a higher P-gp function than frontal cortex and cerebellum.

The study also described simplified quantification methods. SUV values do not reach a stable value during the 30-min-PET scan and therefore, they should not be used to estimate the P-gp function. Previous studies suggested the use of the negative slope of the whole-brain SUV-TAC as a simplified parameter to measure the P-gp function (39). Since the 30-min SUV-TACs of both tracers did not show any washout, the negative slope could not be calculated. Instead, a positive slope using the first 75 seconds of the PET scan, which may be related to the entry of the tracer in the brain, was calculated and correlated to the kinetic parameters. The results showed a good correlation between the positive slope and the K_1 and V_T of both tracers. Thus, the positive slope may be used as a surrogate parameter to estimate the P-gp function, avoiding the blood sampling and full kinetic analysis. This method may facilitate the assessment of the P-gp function in clinical studies.

Although both tracers were able to measure decreases in the P-gp function at the BBB of non-human primates, the use of fluorine-18 for the radiolabeling of [¹⁸F]MC225 may be advantageous. The longer half-life of fluorine-18 ($T_{1/2}=110$ min) compared to carbon-11 ($T_{1/2}=20$ min) allows the distribution of the tracer to remote PET centers and enables accurate plasma measurements. Furthermore, the lower maximum energy of the ¹⁸F isotope provides higher spatial resolution and thus higher quality of PET images (50,51).

5. Conclusion

This head-to-head comparison between [¹⁸F]MC225 and (R)-[¹¹C]verapamil demonstrates that V_T calculated using 30-min scan duration may be a more adequate parameter than K_1 to measure decreases in the P-gp function with both tracers. Although K_1 was selected as the best parameter to measure the P-gp function in previous publications (35,36), the present study found that K_1 was

not different between tracers at baseline conditions, and therefore K_1 could not be used to detect differences in affinity of strong and weak substrates towards the P-gp transporter. Moreover, K_1 values of (*R*)-[^{11}C]verapamil were higher than those from [^{18}F]MC225 in after-inhibition scans, suggesting that (*R*)-[^{11}C]verapamil K_1 values may be affected by other non-specific unknown factors in the brain. The results from both tracers indicate that subcortical regions may present a higher P-gp function than frontal cortex and cerebellum. The higher baseline V_T of [^{18}F]MC225 may allow the quantification of increases in the P-gp function and may facilitate the baseline image registration and fusion to an anatomical image (MRI or CT) (Supplemental Figure 4). Thereby, [^{18}F]MC225 has the potential to become the first radiofluorinated tracer able to measure both decreases and increases in P-gp function at the BBB. Nevertheless, a first-in-man study is required to verify the properties of [^{18}F]MC225 before the tracer can be clinically applied.

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7. Supporting information

Plasma Kinetics and Metabolism Analysis

No significant differences between [^{18}F]MC225 and (*R*)-[^{11}C]verapamil were found in the SUV-TAC of whole-blood neither in baseline nor in after-inhibition scans. However, the metabolite-corrected SUV-TAC of plasma was higher for [^{18}F]MC225 (SUVmean baseline: 2.88 ± 0.28 and after-inhibition: 2.98 ± 0.20) than for (*R*)-[^{11}C]verapamil (SUVmean baseline: 2.00 ± 0.35 and after-inhibition: 1.98 ± 0.32) ($p < 0.001$ in both cases).

No significant differences were found in the parent fraction TACs of both tracers at baseline or after-inhibition. At 30-min after tracer injection, the mean percentage of parent (*R*)-[^{11}C]verapamil was (mean $\pm$ SD) 56.8 ± 6.9 % at baseline and 43.8 ± 8.2 % after P-gp inhibition. A similar decrease of the parent fraction of [^{18}F]MC225 was observed, from 53.2 ± 2.2 % at baseline to 47.4 ± 5.6 % after P-gp inhibition. Supplemental Figure 1 shows the metabolite-corrected plasma SUV-TACs and parent fraction TACs of both tracers in baseline and after-inhibition scans.

The rate constant of elimination of the tracer from the blood (K_e) at baseline was higher for (*R*)-[^{11}C]verapamil ($K_e = 0.0175$) than for [^{18}F]MC225 ($K_e = 0.0155$) ($p = 0.005$). After P-gp inhibition, the K_e values of both tracers were not significantly different (K_e (*R*)-[^{11}C]verapamil = 0.0155 vs K_e [^{18}F]MC225 = 0.0147).

Supplemental Figure 1: Metabolite-corrected SUV-TACs of plasma for both tracers at baseline and after-inhibition (A). Parent fraction of [¹⁸F]MC225 and (R)-[¹¹C]verapamil during the 30-min PET scan (B).

Supplemental Figure 2: K_1 (A), k_2 (B), and V_T (C) values of both tracers in different brain regions, at baseline (left) and after-inhibition (right).

Supplemental Table 1: Regional changes due to the P-gp inhibition in V_T , k_2 , and K_1 relative to the whole brain region in both tracers.

Relative Changes due to P-gp inhibition						
[¹⁸ F]MC225						
Region	Changes in K_1	p-values	Changes in k_2	p-values	Change V_T	p-values
Basal Ganglia	4%	0.035	-5%	0.069	9%	<0.001
Brainstem	7%	0.129	21%	<0.001	-12%	0.079
Cerebellum	-1%	0.618	17%	<0.001	-16%	0.001
Cingulate Cortex	1%	0.776	-2%	0.21	3%	0.042
Hippocampus	-3%	0.004	3%	0.287	-6%	0.019
Hypothalamus	-2%	0.427	1%	0.648	-3%	<0.001
Insular Cortex	3%	0.048	-10%	0.072	14%	0.001
Midbrain	7%	0.085	-3%	0.498	10%	<0.001
Occipital Cortex	-5%	0.016	-3%	<0.001	-2%	0.408
Orbito Frontal Cortex	-2%	0.35	-4%	0.015	2%	0.06
Parietal Cortex	0%	0.971	-4%	0.088	4%	0.112
Striatum	12%	<0.001	0%	0.979	11%	0.002
Temporal Cortex	0%	0.98	1%	0.808	-1%	0.223
Thalamus	16%	<0.001	6%	0.182	9%	<0.001
White Matter	0%	0.917	-3%	0.026	3%	0.119
Relative Changes due to P-gp inhibition						
(R)-[¹¹ C]Verapamil						
Region	Change K_1	p-value	Change k_2	p-value	Change V_T	p-values
Basal Ganglia	9%	<0.001	11%	<0.001	-2%	0.268
Brainstem	17%	<0.001	21%	<0.001	-3%	0.558
Cerebellum	2%	0.168	-4%	<0.001	6%	0.004
Cingulate Cortex	0%	0.932	-10%	<0.001	12%	<0.001
Hippocampus	3%	0.6	12%	<0.001	-8%	0.025
Hypothalamus	9%	<0.001	5%	0.385	3%	0.487
Insular Cortex	7%	<0.001	-4%	0.06	11%	<0.001
Midbrain	27%	<0.001	11%	0.006	14%	<0.001
Occipital Cortex	-4%	0.326	-7%	<0.001	3%	0.437
Orbito Frontal Cortex	-16%	0.021	0%	0.765	-16%	0.026
Parietal Cortex	-4%	<0.001	-2%	0.307	-2%	0.166
Striatum	13%	<0.001	8%	0.001	4%	0.029
Temporal Cortex	3%	0.403	6%	0.014	-3%	0.074

Thalamus	15%	0.004	7%	0.003	8%	0.051
White Matter	4%	0.062	-1%	0.325	4%	<0.001

Supplemental Figure 3: Linear regression analysis of positive slope and the kinetic parameters obtained with 1-TCM.

Supplemental Figure 4: Example of the fusion of the PET images of a subject to its corresponding MRI.

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Chapter 7

A new approach to produce [^{18}F]MC225 via one-step synthesis, a PET radiotracer for measuring P-gp function

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Abstract:

Background: [^{18}F]MC225 is a radiotracer for imaging P-glycoprotein (P-gp) function at the blood-brain barrier. The P-gp function can be altered due to different factors, for instance, decreased P-gp function has been described in patients with Alzheimer's or Parkinson's Disease. The current applied radiosynthesis of [^{18}F]MC225 involves 2 steps, including the distillation of the [^{18}F]fluoroethylbromide intermediate. To develop a more robust synthetic procedure, it is of interest to produce the radiotracer via a 1-step synthesis. The present study describes a new synthetic approach to produce [^{18}F]MC225 via direct ^{18}F -fluorination. Moreover, we also provide the appropriate conditions for the automation of the synthesis. A mesylate precursor was synthesized via a multi-step synthetic route and used for the radiolabeling. The nucleophilic substitution of the mesylate group by [^{18}F]Fluoride was automated in two different synthesis modules: IBA Synthera and Eckert and Ziegler PharmTracer (E&Z). **Results:** The mesylate precursor was synthesized in 7 steps starting with 5-hydroxy-1-tetralone (commercially available) in practical yields. The stability of the precursor was improved via mesylate salt formation method. The radiolabeling was done by adding the mesylate precursor dissolved in DMF to the dried [^{18}F]KF/ $\text{K}_{2.2.2}$ complex and heating at 140°C for 30 min. Quality control by UPLC confirmed the production of [^{18}F]MC225 with a molar activity (A_m) higher than 100 GBq/micromole. The synthesis time in Synthera was 106 min and the product was obtained with a radiochemical purity higher than 95% and RCY of 6.5%, while the production in E&Z lasted 120 min and the product had a lower radiochemical purity (91%) and RCY (3.8%). **Conclusions:** [^{18}F]MC225 was successfully produced via a 1-step reaction. The procedure is suitable for automation using commercially available synthesis modules. The automation of the radiosynthesis in the Synthera module allows the production of the [^{18}F]MC225 by a reliable and simple method.

1. Background

[^{18}F]MC225 is a radiotracer for imaging P-glycoprotein (P-gp) function at the blood-brain barrier (BBB) (1,2). P-gp is an efflux transporter located in the luminal side of the cerebral endothelial cells which constitute the main component of the BBB (3,4). P-gp belongs to the ATP-Binding Cassette transporter family, their function is ATP-dependent and apart from the BBB, these transporters are also located in several tissues involved in absorption and excretion functions such as the intestine, testes, placenta, kidneys, and liver (5). In the BBB, the main function of P-gp is to transport a wide variety of substances out of the brain to the blood. Therefore, this transporter contributes to limit the permeability of the BBB and protects the Central Nervous System (CNS) from neurotoxic compounds (5).

However, the P-gp function can be altered due to different factors. Many unrelated xenobiotic compounds can increase the P-gp expression and function which leads to reduced concentration of drugs in the desired target causing decreases in drug efficacy (6–10). Moreover, dysfunctions in P-gp function have been observed in various disease conditions (5). For instance, patients with intractable epilepsy, have shown increased P-gp expression and function in isolated brain capillaries (11,12). Also, a decreased P-gp function has been reported in Alzheimer's and Parkinson's disease (13). Therefore, the assessment of P-gp function using Positron Emission Tomography (PET) imaging can help to improve the diagnosis of certain CNS diseases where the P-gp function is altered. In addition, this technique can also evaluate treatments that affect P-gp function (14).

[^{18}F]MC225 has been developed as a weak substrate of the P-gp transporter (1). Thus, this radiotracer showed a higher brain uptake at baseline conditions (2), which allows us to measure both increased and decreased P-gp function at the BBB in rats (2). Previous evaluation of the radiotracer in vivo has shown good pharmacokinetic properties, an adequate signal-to-noise ratio, high sensitivity toward the target, and low levels of radio-metabolites inside the brain. All these characteristics make [^{18}F]MC225 a suitable radiotracer (2,15) and worthwhile to perform clinical studies to evaluate the P-gp function in the human brain.

For the validation of [^{18}F]MC225 for clinical use, the production must be compliant with Good Manufacturing Practice (GMP). The currently used GMP-synthesis of [^{18}F]MC225 (Figure 1) implies two synthesis steps, including a distillation step of the [^{18}F]fluoroethylbromide intermediate. Unfortunately, this relatively time-consuming and complex procedure results in low radiochemical yields (1). Thus, it may be convenient to simplify the radiotracer synthesis via 1-step procedure to make the reaction simpler, and thus more robust.

Therefore, this study aims to produce [^{18}F]MC225 via a 1-step reaction using a new MC225 precursor compound which allows the production of the radiotracer by direct ^{18}F -fluorination. Moreover, the study discusses the most appropriate conditions for the automation of the synthesis.

Figure 1: the 2-step synthesis of [^{18}F]MC225.

2. Methods

2.1. Synthesis of phenol precursor (Figure 2)

2.1.1. Synthesis of 5-(benzyloxy)-3,4-dihydronaphthalen-1(2H)-one (2):

5-[3-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-propyl]-5,6,7,8-tetrahydro-naphthalen-1-ol, called the phenol precursor was synthesized as previously described (16) but with some modifications on the synthesis route (Figure 2). 5-Hydroxy-1-tetralone is commercially available (Combi Blocks) and was protected with a benzyl group as follow: 5-Hydroxy tetralone (**1**, 5.0 g, 30.9 mmol, 1.0 eq.) and potassium carbonate (8.5 g, 61.7 mmol, 2.0 eq.) dissolved in ACN were stirred for 15 minutes at room temperature under N_2 -atmosphere. Benzyl bromide (4.04 mL, 33.9 mmol, 1.1 eq.) was added and the resulting beige suspension was stirred overnight at room temperature under N_2 -atmosphere. The reaction mixture was concentrated in vacuum and the crude material was partitioned between water (50 mL) and DCM (50 mL). The layers were separated, and the organic layer was successively washed with water (50 mL), a saturated aqueous solution of NaHCO_3 (50 mL), and brine (50 mL). The organic layer was collected, dried with anhydrous Na_2SO_4 , filtered, and concentrated in vacuum to give a beige oil which slowly solidified on standing. The solids were triturated with heptane (30 mL) to give 5-(benzyloxy)-

3,4-dihydronaphthalen-1(2H)-one (**2**) as a crystalline white solid (6.5 g, 83% of yield). $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ 7.68 (dd, $J = 7.9, 1.1$ Hz, 1H), 7.54 – 7.18 (m, 7H), 7.08 (dd, $J = 8.1, 1.2$ Hz, 1H), 5.11 (s, 2H), 2.98 (t, $J = 6.2$ Hz, 2H), 2.64 (dd, $J = 7.5, 5.7$ Hz, 2H), 2.27 – 1.97 (m, 2H).

2.2.2. Synthesis of 8-(benzyloxy)-4-(3-chloropropyl)-1,2-dihydronaphthalene and (E)-5-(benzyloxy)-1-(3-chloropropylidene)-1,2,3,4-tetrahydronaphthalene (**3**):

For the next step, the benzylated product (compound **2**) was reacted with freshly prepared Grignard reagent. Magnesium turnings (0.96 g, 39.7 mmol, 2.0 eq.), anhydrous THF (30 mL), and iodine (1 grain, catalytic) were combined in a flame-dried three-necked flask under N_2 -atmosphere. Cyclopropyl bromide (2.4 mL, 39.7 mmol, 2.0 eq.) was added portion-wise (total addition time = 15 minutes). After the complete addition of the bromide, the mixture was slowly heated to 55°C . The color of the mixture turned from brown to colorless accompanied by slight effervescence. The resulting mixture was stirred at 55°C for 2h at room temperature under N_2 -atmosphere. At this point, the color of the reaction turned yellowish and the magnesium turnings were completely consumed. The Grignard reagent (CypPrMgBr) was cooled to 0°C by using an ice bath and compound **2** (5.0 g, 19.8 mmol, 1.0 eq.) dissolved in anhydrous THF (30 mL) was added. The resulting mixture was stirred at reflux overnight under N_2 -atmosphere. The mixture was then quenched with a saturated solution of NH_4Cl (100 mL). Diethyl ether (100 mL) was added, and the mixture was stirred for 15 minutes at room temperature. The layers were separated, and the aqueous layer was extracted with diethyl ether (1×25 mL). The organic layers were combined, washed with brine (50 mL), collected, dried with anhydrous Na_2SO_4 , filtered, and concentrated in vacuum to give a yellow oil (6.4 g). The crude was used as such in the next step without purification.

After the Grignard reaction, the cyclopropyl ring was opened by using hydrochloric acid in acetic acid. Thus, the crude material was dissolved in glacial acetic acid (100 mL) and a 20% aqueous solution of HCl (100 mL) was added. The resulting solution was stirred for 2h at room temperature. The acidic mixture was then concentrated in vacuum and the crude was dissolved in DCM (100 mL). The solution was washed with a saturated solution of NaHCO_3 (3×100 mL), water (100 mL), and brine (25 mL). The organic layer was collected, dried with anhydrous Na_2SO_4 , filtered, and concentrated in vacuum to give 8-(benzyloxy)-4-(3-chloropropyl)-1,2-dihydronaphthalene and (E)-5-(benzyloxy)-1-(3-chloropropylidene)-1,2,3,4-tetrahydronaphthalene (**3**) as a brown oil (5.4 g, 87% of yield). No purification was performed regarding the presence of isomers and thus, the isolated material was used as such in the next step.

2.2.3. Synthesis of (Z)-2-(3-(5-(benzyloxy)-3,4-dihydronaphthalen-1-yl)propyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline and (E)-2-(3-(5-(benzyloxy)-3,4-dihydronaphthalen-1(2H)-ylidene)propyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline mixture (4):

The crude mixture containing compound **3** (5.4 g, 19.8 mmol, 1.0 eq.) was dissolved in DMF (100 mL). Anhydrous Na₂CO₃ (6.3 g, 59.4 mmol, 3.0 eq.) was added along with commercially available (Combi Blocks) 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (5.75 g, 29.7 mmol, 1.5 eq.). The mixture was stirred overnight at 100°C under N₂-atmosphere. The mixture was allowed to cool down to room temperature. The mixture was partitioned between ethyl acetate (250 mL) and water (250 mL). The organic layer was collected, washed with water (3× 100 mL), collected, dried with anhydrous Na₂SO₄, filtered, and concentrated in vacuum to give (Z)-2-(3-(5-(benzyloxy)-3,4-dihydronaphthalen-1-yl)propyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline and (E)-2-(3-(5-(benzyloxy)-3,4-dihydronaphthalen-1(2H)-ylidene)propyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline mixture (**4**) compounds as a brown oil (8.2 g, quantitative). No purification was performed due to the presence of isomers and thus, the isolated material was used as such in the next step.

2.2.4. Synthesis of 5-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propyl)-5,6,7,8-tetrahydronaphthalen-1-ol (5):

Subsequently, a hydrogenation step was carried out to reduce double bonds of both isomers and to cleave the benzyl protecting group in one step. Therefore, the isomeric mixture **4** (8.2 g, 17.5 mmol, 1.0 eq.) was dissolved in a mixture of absolute ethanol (100 mL) and DMF (100 mL). This mixture was stirred under N₂-atmosphere for 15 minutes. To this mixture was added, palladium on carbon (Pd/C) (10wt% loading, 1.86 g, 1.75 mmol, 0.1 eq.). The resulting mixture was stirred for 48h at room temperature under H₂-atmosphere (atmospheric pressure) to remove the benzyl group. The mixture was filtered over a thin path of Celite and the filtrate was concentrated in vacuum, dissolved in EtOAc (100 mL), washed with water (4× 100 mL), and brine (100 mL). The organic layer was collected, dried with anhydrous Na₂SO₄, filtered, and concentrated in vacuum to give the crude containing the phenol. The crude was purified via silica column chromatography (DCM: MeOH, 95:5). The fractions that contain the pure compound were concentrated in vacuum to afford 5-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propyl)-5,6,7,8-tetrahydronaphthalen-1-ol (**5**) compound as an off-white foam (1.82 g, 25% yield calculated from benzylated intermediate (**2**)). ¹H NMR (300 MHz, CDCl₃) δ 6.98 (t, J = 7.8 Hz, 1H), 6.77 (d, J = 7.7 Hz, 1H), 6.62 – 6.49 (m, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.57 (s, 2H), 2.88 – 2.45 (m, 9H), 1.92 – 1.57 (m, 8H).

2.2.5. Synthesis of non labelled MC225:

Compound **5** (50.0 mg, 1 eq, 131 μmol) was dissolved in DMF (3 mL) and sodium hydride (15.7 mg, 5 eq, 655 μmol) was added to the dissolved compound. The resulting mixture was stirred for 20 min until no further gas evolution was observed. 1-Bromo-2-fluoroethane (33.3 mg, 20 μL , 2 eq, 262 μmol) was added drop-wise to the reaction mixture. The reaction was monitored by LC-MS. After 20 min of stirring at room temperature, the reaction was complete. The reaction mixture was quenched by addition to a mixture of water/ ethylacetate (50 mL/50 mL). The organic layer was separated and the aqueous layer extracted with ethyl acetate (2x100 mL). The combined organic layers were dried over sodium sulfate and evaporated under reduced pressure. The crude material was purified by automated column chromatography yielding non labelled **MC225** (35.0 mg, 62.5 %). ^1H NMR (299 MHz, CDCl_3) δ 7.08 (t, $J = 7.9$ Hz, 1H), 6.84 (d, $J = 7.8$ Hz, 1H), 6.63 (d, $J = 8.1$ Hz, 1H), 6.59 (s, 1H), 6.52 (s, 1H), 4.90 – 4.77 (m, 1H), 4.75 – 4.57 (m, 1H), 4.24 (dd, $J = 5.0, 3.4$ Hz, 1H), 4.20 – 4.10 (m, 1H), 3.84 (s, 3H), 3.83 (s, 3H), 3.56 (s, 2H), 2.93 – 2.76 (m, 3H), 2.76 – 2.63 (m, 3H), 2.58 – 2.46 (m, 2H), 1.98 – 1.52 (m, 9H).

Figure 2: Alternative route for synthesizing the phenol precursor.

2.3. Synthesis mesylate precursor (Figure 3)

2.3.1. Synthesis of 2-(3-(5-(2-(benzyloxy)ethoxy)-1,2,3,4-tetrahydronaphthalen-1-yl)propyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (6):

5-(3-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propyl)-5,6,7,8-tetrahydronaphthalen-1-ol (**5**) (150.0 mg, 1 eq, 393.2 μmol) (**phenol precursor** or **5**) was dissolved in DMF (12 mL) and sodium hydride (47.18 mg, 5 eq, 1.97 mmol) was added to the dissolved compound. The resulting mixture was stirred for 2 hours until no gas evolution appeared anymore. ((2-

Bromoethoxy)methyl)benzene (169 mg, 0.124 mL, 1.99 eq, 784 μmol) was added drop-wise to the reaction, and the progress of the reaction was monitored by LC-MS. After 20 min of stirring the reaction was completed according to LC-MS. The reaction mixture was quenched by adding to a mixture of water/ ethyl acetate (50 mL/50 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (2x100 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The compound was analyzed by HPLC-MS. The crude material was purified by automated column chromatography (Biotage Purification System, Uppsala, Sweden) with a gradient of 0% to 50% ethyl acetate in heptane yielding 2-(3-(5-(2-(benzyloxy)ethoxy)-1,2,3,4-tetrahydronaphthalen-1-yl)propyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (**6**) (165 mg, 84.4%). ^1H NMR (300 MHz, CDCl_3) δ 7.43 – 7.28 (m, 5H), 7.07 (t, J = 7.9 Hz, 1H), 6.81 (d, J = 7.7 Hz, 1H), 6.67 – 6.61 (m, 1H), 6.59 (s, 1H), 6.52 (s, 1H), 4.65 (s, 2H), 4.14 (dd, J = 5.8, 4.1 Hz, 2H), 3.90 – 3.78 (m, 8H), 3.55 (s, 2H), 2.91 – 2.76 (m, 3H), 2.76 – 2.62 (m, 4H), 2.58 – 2.44 (m, 2H), 1.91 – 1.60 (m, 8H).

2.3.2. Synthesis of 2-((5-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propyl)-5,6,7,8-tetrahydronaphthalen-1-yl)oxy)ethan-1-ol (**7**):

A solution of **6** (150.00 mg, 1 eq., 290.87 μmol) in methanol (12 mL) and acetic acid (0.5 mL) was stirred under N_2 for 20 min. Pd/C (20.00 mg, 0.5 Eq, 29.09 μmol) was added to the solution and the reaction was stirred overnight at 5 bar H_2 pressure. After 3 days of stirring full conversion was observed. The mixture was filtered through a pad of Celite and the organic solvents were removed by rotary evaporation. The residue was dissolved in ethyl acetate and washed with a potassium bicarbonate solution. The organic layer was dried over anhydrous Na_2SO_4 , filtered, and evaporated under reduced pressure yielding 2-((5-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propyl)-5,6,7,8-tetrahydronaphthalen-1-yl)oxy)ethan-1-ol (**7**) (100.20 mg, 81 %). ^1H NMR (299 MHz, cdcl_3) δ 7.08 (t, J = 7.9 Hz, 1H), 6.84 (d, J = 7.8 Hz, 1H), 6.65 (d, J = 8.0 Hz, 1H), 6.59 (s, 1H), 6.52 (s, 1H), 4.07 (dd, J = 5.2, 3.7 Hz, 2H), 3.97 (dd, J = 5.2, 3.6 Hz, 2H), 3.84 (s, 3H), 3.83 (s, 3H), 3.56 (s, 2H), 2.89 – 2.76 (m, 3H), 2.76 – 2.65 (m, 3H), 2.57 – 2.46 (m, 2H), 1.93 – 1.51 (m, 9H).

2.3.3. Synthesis of 6,7-dimethoxy-2-(3-(5-(2-((methylsulfonyl)oxy)ethoxy)-1,2,3,4-tetrahydronaphthalen-1-yl)propyl)-1,2,3,4-tetrahydroisoquinolin-2-ium methanesulfonate (**8**):

Compound **7** (105.00 mg, 1 eq., 246.73 μmol) was used without further purification and was dissolved in 1,4-dioxane (8 mL) and methanesulfonic anhydride (45.13 mg, 1.05 eq., 259.06 μmol) was added to the mixture. The resulting mixture was heated to reflux during one week until the reaction was complete. The reaction mixture was evaporated under reduced pressure, and 6,7-dimethoxy-2-(3-(5-(2-((methylsulfonyl)oxy)ethoxy)-1,2,3,4-tetrahydronaphthalen-1-yl)propyl)-

1,2,3,4-tetrahydroisoquinolin-2-ium methanesulfonate (**8**) MC225 precursor was isolated as its mesylate salt. At this stage, we could not isolate intermediate **8** in pure form (93 mg, 75 % pure,) and decided to use it as such for the radiolabeling.

Figure 3: Synthesis of the MC225 mesylate precursor as its mesylate salt.

2.4. [^{18}F]Fluoride production

[^{18}F]Fluoride was produced using a cyclotron (IBA Cyclone, Louvain-la-Neuve, Belgium) by the nuclear reaction $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$.

Figure 4: One-step radiosynthesis of [^{18}F]MC225 using the MC225 mesylate precursor.

2.5. Automated synthesis (Figure 4)

The synthesis was automated using two different modules the IBA Synthra module (Figure 5) and the Modular Lab PharmTracer from Eckert & Ziegler (Figure 6). Aqueous [^{18}F]fluoride was trapped on Sep-Pak Light Accell Plus QMA anion exchange cartridge (Waters, Milford, USA) previously conditioned with 5 ml of Na_2CO_3 1.4%, washed with 10 ml of H_2O for injection, and then dried with a nitrogen stream. [^{18}F]Fluoride was eluted using a solution of 1.3 ± 0.12 mg K_2CO_3 and 7.1 ± 0.26 mg Kryptofix 222 ($\text{K}_{2.2.2}$) in 1 ml of $\text{ACN}/\text{H}_2\text{O}$ (8:2). Azeotropic drying of the [^{18}F]KF/ $\text{K}_{2.2.2}$ complex was performed at 110°C under an argon flow and adding 1 ml of ACN . After the evaporation of the solvent, the procedure was repeated twice using 0.5 ml of ACN .

Immediately after the azeotropic drying, 1 mg of mesylate precursor dissolved in 1 ml DMF (or 0.5 ml in the case of E&Z) was added to the reactor vial containing the dried [^{18}F]KF/ $\text{K}_{2.2.2}$ complex. The reaction was heated at 140°C for 30 min and after cooling down the solution 1 ml of H_2O was added (1.3 ml in case of E&Z). The crude reaction was injected onto the semi-preparative HPLC and the product was collected after 12 min.

Purification of the crude reaction was done in a semi-preparative HPLC system using a Symmetryshield RP8 $5\mu\text{m}$ 7.8×300 mm column. 0.1 M NaOAc/ACN (5.5/4.5) (v/v) ($\text{pH} = 4.7$) were used as eluent at a flow of 3 ml/min. The UV signal was measured at a wavelength of 215 nm.

Figure 5: Scheme IBA Synthra module for the 1-step synthesis of [^{18}F]MC225.

Figure 6: Scheme of Eckert & Ziegler module for the synthesis of [^{18}F]MC225 using a 1-step procedure.

2.6. Formulation of the product

Formulation of the product was done as previously described (1). Briefly, the desired product eluted at 12 min from the Prep-HPLC was collected in 60 ml of sterile H_2O in an 80 ml bottle. The mixture was mixed with Helium and the mixture was passed through an Oasis HLB 1 cm^3 (30 mg) extraction cartridge where the product was trapped. The cartridge was washed twice with 8 ml sterile H_2O and the product was eluted with 1 ml of ethanol. Next, 4 ml of 0.9% NaCl was passed through the cartridge to formulate the final product. The solution was filtered through a Millipore Millex LG Filter (0.2 μm) before collection in a sterile vial.

2.7. Quality control methods

Quality control was executed with a Waters Acquity H-class UPLC system (Milford, CT, USA) as previously described (2). The system used a Berthold FlowXStar LB 513 as a radioactivity detector (Bad Wildbad, Germany) and a Waters Acquity UPLC BEH Shield RP18 1.7 μm (3.0mmx50mm) column. The product eluted after 3.5 minutes using ACN / 10 mM NH_4CO_3 (pH=9.5) (50/50) at a flow rate of 0.8 ml/min. The UV detection was set to 215 nm. The reference compound (non-labelled MC225) was used to prepare a calibration curve to know the amount of

non-labelled compound and thus the molar activity (A_m) of the final product at the end of the radiosynthesis.

3. Results and Discussion

This study aimed to develop a new synthesis method to produce the PET radiotracer [^{18}F]MC225 via a 1-step synthesis. To this purpose, a mesylate precursor has been developed to facilitate direct ^{18}F -fluorination yielding [^{18}F]MC225.

This mesylate precursor (**8**) was synthesized from the phenol precursor (**5**) which was previously used in the 2-step synthesis of [^{18}F]MC225 (*1*). Phenol precursor (**5**) was produced via an alternative synthesis which uses a benzyl-protected tetralone (**2**). The benzyl protecting group was chosen to reduce both the double bonds of both isomers (**4**) and to cleave the benzyl protecting group in one step. Palladium on carbon (Pd/C) was used as a catalyst and the hydrogenation was performed at atmospheric pressure. Full reduction of the double bonds was observed after stirring the mixture for 2 h at room temperature. The reaction was run for 48h to remove the benzyl group. Moreover, the benzylated species were more stable than the unprotected phenol. At the end of the synthesis, the phenol precursor (**5**) was isolated and purified via column chromatography and obtained in a 25% yield (calculated from intermediate **2**, Figure 2).

The development of the mesylate precursor (**8**) was challenging. Firstly, the deprotection of the benzyl group of compound **6** was facing difficulties. Compound **6** seems to be unstable on silica thus reaction needs to achieve quantitative conversion to continue with the next step without further purification. The reaction was slow and different batches of Pd/C and Pd/C hydroxide were added to speed up the reaction and to obtain a maximum conversion. The reaction took more than one week to reach full conversion. Moreover, the reaction mixture needed to be slightly acidic to be able to reach completion, thus acetic acid was added. In the following step, compound **7** was used without further purification, and therefore, it was dissolved in dioxane and reacted with methanesulfonic anhydride at reflux. However, the mesylate precursor was not stable. Especially basic workup of the reaction mixture resulted in the elimination of the mesylate. Several strategies were explored to stabilize compound **8**. Eventually, it was observed that the mesylate salt formed during reaction with methanesulfonic anhydride was reasonably stable and helped to stabilize the precursor for a longer time. However, the final mesylate precursor showed several impurities (purity 75%). Therefore, several attempts were made to improve purity e.g., via RP-automated column chromatography, prep HPLC and precipitation. All these attempts were unsuccessful leading to the deterioration of the product on the column or the isolation of impure product in very low yield after precipitation. Thus, the crude reaction mixture was evaporated and used as such for the labeling experiments.

The radiolabeling was performed by adding the mesylate precursor to the dried [^{18}F]KF/K_{2.2.2} complex and heating at 140 °C for 30 minutes. After the radiochemistry reaction, the crude mixture was purified using semi-preparative HPLC (as described in the methods) (Figure 7).

Figure 7: (A) Crude reaction (30 min at 140°C), the product appeared after 10-11 minutes injection and (B) retention of reference compound MC225 (10-11 min).

The automated synthesis was performed using two modules: IBA Synthera Synthesis Module and the Modular Lab PharmTracer (Eckert & Ziegler). The best results were obtained with IBA Synthera module. The final product produced by both modules was analyzed by UPLC. Table 1 shows the results of both synthesis modules. The total synthesis time was 20 min shorter in the Synthera module than with E&Z module, mainly because of the faster drying process of the [^{18}F]KF/K_{2.2.2} complex. The radiochemical purity of [^{18}F]MC225 and the radiochemical yield (RCY) corrected for decay was higher in the IBA module. However, a high A_m of the final product was obtained with both modules. Regarding the preparation for the synthesis, the set-up of the synthesis was easier and quicker in the IBA Synthera than in the E&Z module. Although the Modular Lab PharmTracer from E&Z allows the configuration of multi-step and complicated

synthesis by the combination of various disposable components, the amount of tubing and valves make the preparation more laborious. In the case of Synthra, the cartridge provides the connections and only the reagents must be placed in the right position during the preparation, thus the time spent in the preparation is shorter. For this reason, Synthra may be a preferable module to perform this synthesis. Independently of the module, we could produce a dose of 407 ± 263 MBq of [^{18}F]MC225 in a single run starting from 15-20 GBq of [^{18}F]fluoride, which means we could perform multiple animals per day.

Table 1: Characteristics of the 1-step synthesis performed in Eckert & Ziegler and IBA Synthra. Values reported in mean $\pm$ SD

Procedure	Total Reaction time (min)	Drying time (min)	RCY (%)	% Area [^{18}F]MC225 (UPLC)	Molar Activity (A_m) (GBq/ mmol)
Eckert & Ziegler (n=9)	120.44 ± 26.33	20.33 ± 5.89	3.76 ± 2	91.70 ± 3.8	> 100000
IBA synthra (n=9)	106.09 ± 13.06	10.44 ± 0.73	6.53 ± 3	97.14 ± 1.12	> 100000

The final product collected from the semi-preparative HPLC was injected onto the UPLC system to perform quality control. Figure 8 shows an example of UPLC chromatography that shows the desired product appearing at 3.5 min after the injection.

Figure 8: Final product injected into the UPLC system. [^{18}F]MC225 elutes at 3.5 min.

This study provides an alternative method for the production of [^{18}F]MC225 using a 1-step approach. However, to obtain a GMP compliant synthesis a suitable purification of the mesylate precursor is still warranted. Nevertheless, an automated synthesis using the mesylate precursor and the IBA Synthera module produced [^{18}F]MC225 in a reliable and simple manner. Quality control performed with UPLC ensures an adequate purity and A_m of the final product. Moreover, the 1-step synthesis may be a more attractive method for the translation of the synthesis to other PET centers.

4. Conclusion

Overall, the production of [^{18}F]MC225 by the 1-step synthesis using the mesylate precursor and the IBA Synthera module seems to be the most successful automated method. The highest radiochemical yield and the adequate purity and A_m of the final product will enable the use of this procedure for GMP productions.

5. List of abbreviations

Acetonitrile: ACN

Ammonium chloride: NH_4Cl

Blood-brain barrier: BBB

Central Nervous System: CNS

Cyclopropyl magnesium bromide: CypPrMgBr

Dichloromethane: DCM

Dimethylformamide: DMF

Ethyl acetate: EtOAc

Good Manufacturing Practice: GMP

High-performance liquid chromatography-mass spectroscopy: HPLC-MS

Hour: h

Hydrochloric acid: HCl

Hydrogen atmosphere: H_2 -atmosphere

Liquid chromatography-mass spectroscopy: LC-MS

Methanol: MeOH

Micrometer: μm

Milligram: mg

Milliliter: ml

Minute: min

Molar activity: A_m

Nanometer: nm

Nitrogen atmosphere: N_2 -atmosphere

Palladium on carbon: Pd/C

P-glycoprotein: P-gp

Positron Emission Tomography: PET

Potassium carbonate: K_2CO_3

Proton nuclear magnetic resonance: H-NMR

Radiochemical yield: RCY

Reverse Phase automated column chromatography: RP-automated column chromatography

Sodium acetate: NaOAc

Sodium bicarbonate: NaHCO_3

Sodium carbonate: Na_2CO_3

Sodium chloride: NaCl

Sodium sulfate: Na_2SO_4

Tetrahydrofuran: THF

Thin Layer Chromatography: TLC

Ultra-performance liquid chromatography: UPLC

Ultraviolet: UV

Water: H_2O

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Chapter 8

***In vivo* induction of P-glycoprotein function can be measured with [¹⁸F]MC225 and PET**

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Abstract

P-glycoprotein (P-gp) is an efflux pump located at the blood-brain barrier (BBB) that contributes to the protection of the central nervous system by transporting neurotoxic compounds out of the brain. A decline in P-gp function has been related to the pathogenesis of neurodegenerative diseases. P-gp inducers can increase the P-gp function and are considered as potential candidates for the treatment of such disorders. The P-gp inducer MC111 increased P-gp expression and function in SW480 human colon adenocarcinoma and colo-320 cells, respectively. Our study aims to evaluate the P-gp inducing effect of MC111 in the whole brain *in vivo*, using the P-gp tracer [¹⁸F]MC225 and Positron Emission Tomography (PET). Eighteen Wistar rats were treated with either vehicle solution, 4.5 mg/kg of MC111 (low-dose group), or 6 mg/kg of MC111 (high-dose group). Animals underwent a 60-minute dynamic-PET scan with arterial-blood sampling, 24 hours after treatment with the inducer. Data were analyzed using the 1-Tissue-Compartment-Model and metabolite-corrected plasma as input function. Model parameters as the influx constant (K_i) and volume of distribution (V_T) were calculated, which reflect the *in vivo* P-gp function. P-gp and Pregnane Xenobiotic Receptor (PXR) expression of the whole brain was assessed using western blot. The administration of MC111 decreased K_i and V_T of [¹⁸F]MC225 in the whole-brain and all the selected brain regions. In the high-dose group, whole-brain K_i was decreased by 34% ($K_{i\text{-high-dose}} = 0.20 \pm 0.02$ vs $K_{i\text{-control}} = 0.30 \pm 0.02$; $p < 0.001$) and in the low-dose group by 7% ($K_{i\text{-low-dose}} = 0.28 \pm 0.02$ vs $K_{i\text{-control}} = 0.30 \pm 0.02$; $p = 0.42$) compared to controls. Whole-brain V_T was decreased by 25% in the high-dose group ($V_{T\text{-high-dose}} = 5.92 \pm 0.41$ vs $V_{T\text{-control}} = 7.82 \pm 0.38$; $p < 0.001$) and by 6% in the low-dose group ($V_{T\text{-low-dose}} = 7.35 \pm 0.38$ vs $V_{T\text{-control}} = 7.82 \pm 0.37$; $p = 0.38$) compared to controls. k_2 values did not vary after treatment. The treatment did not affect the metabolism of [¹⁸F]MC225. Western blot studies did not detect changes in the P-gp expression. The decrease in K_i and V_T values after treatment with the inducer indicates an increase in the P-gp functionality at the BBB of treated rats. However, no changes in the P-gp expression were detected. The results verify that MC111 induces

the P-gp function *in vivo*. These data also confirm the ability of [¹⁸F]MC225 to measure increases in the P-gp function at the BBB in rats.

Keywords: ABC transporters, Brain Imaging, Efflux transporters, P-glycoprotein, P-gp inducers.

1. Introduction

P-glycoprotein (P-gp) is an efflux transporter that belongs to the ATP-Binding Cassette (ABC) transporters family and is located at the luminal side of the endothelial cells at the blood-brain barrier (BBB) and other blood-tissue barriers (1). P-gp pumps a wide variety of compounds, including endogenous and exogenous substances from the brain to the circulation, by ATP-driven transport (2). Thereby, P-gp located at the BBB contributes to the protection of the brain from harmful substances and maintains the homeostasis needed for the appropriate neural function (3). However, several factors can affect P-gp function or expression such as inflammatory responses, stress, therapeutic drugs, and diet (4).

A decline in the P-gp function at the BBB can increase the concentration of neurotoxic compounds inside the brain, which can be related to the onset and progression of several neurodegenerative diseases such as Alzheimer's (AD). It has been reported that P-gp is involved in the clearance of A β peptides since the inhibition of P-gp decreased extracellular levels of A β secretions *in vitro* (5). Moreover, an inverse correlation between A β deposition and expression of P-gp was found in the BBB of elderly non-demented subjects (6). Also, PET imaging studies performed in AD patients found an increase of [11 C]verapamil uptake inside the brain compared to healthy volunteers, which indicates a decreased P-gp function (7).

Several studies have already reported the use of P-gp inducers to restore the function of P-gp in neurodegenerative diseases (8,9). P-gp induction does not occur via direct binding to the P-gp transporter and P-gp expression and function can be regulated by different mechanisms including the activation of several transcription factors and posttranscriptional and posttranslational mechanisms (10–12). Recently, it has been proposed that an increase in P-gp expression at the cell surface can be caused by relocating intracellular P-gp protein to the plasma membrane (10,11). Among the transcription factors, the nuclear receptors Pregnane Xenobiotic Receptor (PXR) and Constitutive Androstane Receptor (CAR) have been identified as key factors in the P-gp up-regulation (13). The PXR and CAR can be activated by endogenous or exogenous compounds (14). PXR is activated by steroids, chemotherapeutics, HIV protease inhibitors, glucocorticoids, and anticonvulsants but also by dietary compounds and natural steroids such as pregnenolone and progesterone (15). CAR is activated by endogenous ligands such as bile acids but also by drugs, dietary compounds, and pollutants (16).

Since P-gp inducers can play an important role in the treatment of neurodegenerative diseases (8,9) several preclinical studies have already reported the efficacy of such drugs. A study performed in a transgenic mouse model of human AD found a reduced P-gp function in these animals compared to wild-type mice. After treatment with pregnenolone-16 α -carbonitrile for 7 days, PXR was activated, and consequently, the P-gp function was restored resulting in an

increase of A β clearance from the brain (17). 1,25-Dihydroxyvitamin D3 enriched-diet also induced the P-gp function and reduced the A β deposition in mice models of AD. In contrast, AD model mice fed with a vitamin D-deficient diet showed a reduction in P-gp expression and increased A β plaques in the brain as well as increased cognitive deficits (18). The administration of St. John's Wort, which is a herbal drug used in the treatment of depression, to transgenic AD mice enhanced the cerebral P-gp function and reduced A β accumulation (19).

Recently, a new promising compound, 4'-((4-cyclohexylpiperazin-1-yl)methyl)-[1,1'-biphenyl-4-ol) (MC111) has been identified as a P-gp inducer by *in vitro* studies. MC111 activity was assessed in colo-320 cells. After treatment with MC111, an increase in [³H]verapamil efflux was observed, which indicates an increase in the P-gp function. Western blot studies also indicated that MC111 increases P-gp expression in SW480 human colon adenocarcinoma cells (20). Furthermore, this new compound also activates the Breast Cancer Resistance Protein (BCRP) function and expression in colo-320 and MCF7 cells (20). The dual activity of MC111 may be beneficial for the treatment of several neurodegenerative diseases where not only an impaired P-gp function and expression has been observed but also alterations in the BCRP and other ABC transporters (21,22).

This study aims to demonstrate the P-gp induction effect of MC111 *in vivo* at the BBB of healthy rats. For this purpose, we used Positron Emission Tomography (PET) imaging, which is a non-invasive imaging technique that quantifies the biochemical process *in vivo*, and the tracer [¹⁸F]MC225 for measuring the P-gp function. [¹⁸F]MC225 is considered as a weak P-gp substrate and therefore, it shows relatively high uptake in the brain at baseline conditions when the P-gp transporter is completely functional. This characteristic of [¹⁸F]MC225 could be beneficial for imaging increased P-gp function upon treatment with an inducer when the tracer uptake in the brain is expected to decrease (23,24).

In the present study, we will evaluate the effect of an inducer for the first time *in vivo*, after its administration to living subjects. To this aim, a dynamic PET scan will be made in eighteen healthy male rats. The kinetics of [¹⁸F]MC225 will be assessed by the 1-Tissue Compartment model (1-TCM) and changes in the P-gp function will be reflected by the influx constant K₁ and volume of distribution (V_T). Moreover, P-gp expression in brain tissue will be evaluated post-mortem using western blots. The results may yield insight into the potential therapeutic application of MC111 in diseases where the P-gp function/expression is decreased.

2. Experimental Section

2.1. Chemicals

6,7-Dimethoxy-2-(3-(5-(2-((methylsulfonyl)oxy)ethoxy)-1,2,3,4-tetrahydronaphthalen-1-yl)propyl)-1,2,3,4-tetrahydroisoquinolin-2-ium methanesulfonate salt (mesylate [^{18}F]MC225 precursor) was purchased from Syncom (Groningen, the Netherlands). The inducer MC111 (4'-((4-cyclohexylpiperazin-1-yl)methyl)-[1,1'-biphenyl-4-ol]) was supplied by the University of Bari, Italy. Chemicals were purchased from Sigma-Aldrich (St.Louis, MO) and isoflurane from Pharmachemie (Haarlem, the Netherlands).

2.2. Tracer production

[^{18}F]MC225 was synthesized by a one-step reaction (25). Briefly, the radiosynthesis was automated and performed in Synthera[®] modules (IBA RadioPharma Solutions). After drying the [^{18}F]Fluoride, the mesylate [^{18}F]MC225 precursor dissolved in DMF was added to the vial and the mixture was heated at 140°C for 30 min. After purification on a semi-preparative HPLC system, the radiotracer was reformulated to 15% ethanol in sterile 0.9% NaCl solution. Quality control procedures were performed in a UPLC system as previously described (24).

Figure 8: Molecular structures of the *P-gp* tracer [^{18}F]MC225 (A) and the *P-gp* inducer MC111 (B).

2.3. Animals

Eighteen healthy male Wistar rats (RjHan: WI) were obtained from Janvier Labs (France) (281 ± 9 g). Before starting the experiments, rats were acclimatized to the new environment for at least 7 days. Rats were housed in groups of 2 in a temperature-controlled room ($21 \pm 2^\circ\text{C}$) with 12h dark/12h light cycles. Water and standard laboratory chow were available *ad libitum*. All animal experiments were performed in compliance with Dutch and EU regulations. The experimental protocol was approved by the National Committee on Animal Experiments of the Netherlands (CCD, the Hague) and the Institutional Animal Care and Use Committee of the University of

Groningen (CCD license number: AVD105002015166, IvD protocol: 15166-01-002 and CCD license number: AVD1050020198648 IvD protocol: 198648-01-001).

Rats were randomly divided into three groups treated with either vehicle solution (control group, n = 6), MC111 inducer at a dose of 4.5 mg/kg (low-dose group, n = 6), or MC111 inducer at a dose of 6 mg/kg (high-dose group, n = 6). The injection of MC111 was performed 24 hours before the PET scan, intravenously through a tail vein under isoflurane anesthesia (12 ± 9 min). First, MC111 was dissolved in DMSO (5% v/v) (42 μ l) and polyethylene glycol (PEG) 300 (10%) (84 μ l), and subsequently, sterile water (85%) was added to the mixture, resulting in a total volume of 0.85 ml (injection volume).

2.4. PET imaging

One day after the treatment with MC111, animals were transported to the MicroPET facility. Before the PET scans, rats underwent surgery to place a cannula in a side branch of the femoral artery which allows blood sampling during the scan. After the surgery, anesthetized rats were placed in the bed of the PET scanner (Focus 220 MicroPET, Siemens Healthcare, USA) with their head placed in the center of the field of view. First, a transmission scan was acquired using a ⁵⁷Co point source. Then, a 60-min dynamic PET scan was started simultaneously with the tracer injection. Rats were injected with [¹⁸F]MC225 (28.4 ± 10.7 MBq in a total volume of 1 ml) via the tail vein at a speed of 1 ml/min. After the administration of [¹⁸F]MC225, 15 blood samples were taken from the arterial cannula. These blood samples were used for the determination of the radiotracer concentration in blood and plasma and to measure the percentage of [¹⁸F]MC225 and its radioactive metabolites in plasma. Rats were anesthetized with isoflurane in 95% oxygen (induction 5% and maintenance 1.5-2%) during the surgery, the tracer administration, and the PET scan. Body temperature was maintained close to normal values and blood oxygenation and heart rate were continuously monitored during the scan, using pulse oximeters. After the scan, rats were perfused with cold phosphate-buffered saline (PBS) and sacrificed. Brain tissues were immediately collected and stored at -80 °C after flash-freezing in liquid nitrogen.

2.5. Arterial blood sampling and metabolism

Immediately after the injection of the tracer, blood samples were collected at the following time points: 10, 20, 30, 40, and 50 seconds, and 1, 1.3, 2, 3, 5, 7.3, 10, 15, 30, and 60 minutes. The drawn volume of blood (0.1 ml) was replaced by a warm saline solution containing 0.1% heparin. From each blood sample, a 25 μ l sample of whole blood was pipetted into a different tube. The remaining blood was centrifuged for 5 min at 3700 rpm (Mikro 20, Hettich, Germany) and 25 μ l of plasma was transferred to another tube. Radioactivity in whole blood and plasma was measured

in a gamma counter (LKB Wallac, Turku, Finland) and values were corrected for decay from the time of tracer injection.

The [^{18}F]MC225 parent fraction and radio-metabolites in plasma were assessed by Thin-Layer Chromatography (TLC) (F-254 silica gel plates, Sigma–Aldrich) as follows (24). Plasma samples acquired at 10 seconds and 1, 3, 5, 7.3, 10, 15, 30, and 60 minutes were used for metabolite analysis. Fifty μl of ACN was added to the plasma sample to precipitate the proteins. Then, plasma samples were vortexed and centrifuged for 5 minutes at 6000 RPM (Hettich Mikro 20, Hettich Zentrifugen, Germany). The protein-free supernatant (2.5 μl) was spotted onto a silica gel TLC plate. When sample spots were dried, the samples on TLC plates were eluted with 10% of MeOH in EtOAc. TLC plates were exposed to activated phosphor storage plates (PerkinElmer, USA) overnight. The following day, the storage plates were read using a GE Amersham TM Typhoon Molecular Imager (GE Healthcare, Life Science, MA, USA). OptiQuant 03.00 software (PerkinElmer, USA) was used to calculate the fractions of parent tracer and radioactive metabolites.

To calculate the percentage of the radioactive metabolites in plasma, a single exponential equation was fitted to the values obtained from the metabolite analysis, using an iterative non-linear least-squares approach and GraphPad software (GraphPad Prism version 7.02, California, USA): $Y = (Y_0 - \text{plateau}) * \exp(-ke * X) + \text{plateau}$ (26), where Y is the percentage of parent fraction at different time points, Y_0 the intercept which was fixed to 100% (percentage of parent fraction at the beginning of the scan), Ke the elimination constant, and X the time.

2.6. PET image reconstruction and analysis

Data from the transmission scan were used for attenuation and scatter corrections. The list-mode data of the emission scan was reconstructed into 21 frames (6 x 10; 4 x 30; 2 x 60; 1 x 120; 1 x 180; 4 x 300; 3 x 600) using OSEM 2D, four iterations, and 16 subsets. The PET data were normalized and corrected for scattering, attenuation, and radioactivity decay.

PMOD v3.8 software (PMOD Technologies, Zürich, Switzerland) was used to automatically and individually register the PET images to an [^{18}F]MC225 specific template (27,28) by rigid transformation.

The amygdala, cerebellum, corpus callosum, medial geniculate, mesencephalic, septum, superior colliculus, striatum, cortex, hippocampus, hypothalamus, midbrain, brainstem, thalamus, basal ganglia, and a whole-brain region were selected as volumes of interest (VOI) for this study. The radioactivity concentration in each VOI (KBq/ml) was calculated and corrected for injected dose

(KBq) and the bodyweight of the animal (g) and thus, it was expressed as Standardized Uptake Value (SUV).

2.7. Input function analysis

Time-Activity Curves (TAC) of whole-blood, plasma, and plasma-corrected for metabolites were calculated using the blood measurement and expressed in SUV. The metabolite-corrected plasma TAC-SUV was obtained by multiplying the SUV values of the plasma samples by the percentage of parent fraction in each time-point.

The first-order rate constant of elimination (K_e) was calculated by fitting a single exponential curve to the metabolite-corrected plasma TAC-SUV of each subject, by an iterative non-linear least-squares approach using GraphPad software (GraphPad Prism version 7.02, California, USA): $Y = Y_0 \times \exp(-K_e \times X)$, where Y is the metabolite-corrected SUV value in plasma; Y_0 is the Y-intercept; K_e is the first-order rate constant of elimination and X the time (29).

2.8. Pharmacokinetic Modelling

Measured radioactivity in whole-blood and plasma as well as the percentage of [¹⁸F]MC225 parent tracer, were used as input functions for the pharmacokinetic analysis (PMOD Technologies, Zürich, Switzerland).

The 1-Tissue Compartmental Model (1-TCM) has been selected as the model of choice to analyze the [¹⁸F]MC225 data in rats and the cerebral volume of blood was fixed to 5% (24,27). The TACs were corrected for blood delay. First, the blood delay of the whole-brain region TAC was estimated and then this value was fixed for the rest of the brain regions.

The influx rate constant K_1 is considered as the best parameter to measure the P-gp at the BBB (24,30,31), thus the study evaluated the changes in K_1 caused by the administration of the treatment. Since the volume of distribution (V_T) can also be used to assess changes in the P-gp function, this parameter was also calculated (24). Some studies reported changes in the efflux constant k_2 due to the P-gp inhibition (32,33), thus the effect of the treatment on k_2 values was also evaluated.

2.9. Parametric Images

Parametric images representing K_1 , V_T , and k_2 values of three rats from different groups (control, low-dose, and high-dose group) were used for illustrative purposes. To this aim, the metabolite-corrected plasma TAC of each subject was used as input function for 1-TCM analysis ($v_B = 5\%$), using PMOD.

2.10. Western blot analysis

P-glycoprotein (P-gp) and Pregnane X receptor (PXR) expression levels in whole brain tissue were quantified by means of western blot.

Protein extraction was carried out by adding cold RIPA buffer (Sigma, USA) with protease inhibitor cocktail (Sigma, USA) to brain slices in an approximated 10:1 proportion (volume/weight) and tissue disruption was performed in a TissueLyser II (Qiagen, Switzerland). Then, tissue lysates were centrifuged for 30 minutes at 20000 g, and supernatants were collected to prepare the western blot. Protein concentration in the lysates was quantified using a Micro BCA Protein assay kit (ThermoFisher, USA).

The necessary volume of cell lysate containing a total amount of 25 µg of protein was subjected to SDS-PAGE in 4-15% Criterion™ TGX™ Precast Midi Protein Gel (BioRad, USA) using a constant voltage of 140V. Then proteins were transferred onto a PVDF membrane (Millipore, Ireland) using a Trans-Blot semi-dry system (Bio-Rad, USA) with a limited voltage of 25V and 180 milliamps for two hours. After the blotting step, membranes were blocked for 1 hour with a 3% Bovine Serum Albumin (BSA) solution (in Tris-Chloride Buffer with a 0.1% of Tween 20 (TBST)). Once blocked, target proteins were detected through incubation with primary antibodies against P-gp (Rabbit monoclonal to P-glycoprotein ab170904, Abcam, UK), PXR (Rabbit polyclonal to PXR ab192579, Abcam, UK), β -Actin (Mouse monoclonal to β -Actin ab8226, Abcam, UK) and Histone H3 (Rabbit polyclonal to H3, ab1791, Abcam, UK) diluted 1:1000, 1:1000, 1:5000 and 1:1000 respectively in TBS with 3% of BSA. Primary antibodies were incubated overnight at room temperature under agitation, and then membranes were washed three times in TBST to remove the excess of primary antibodies and avoid unspecific signaling. As secondary antibodies, an HRP-conjugated Goat anti-Rabbit IgG (P044801-2, Dako, Denmark) and HRP-conjugated Rabbit anti-Mouse IgG (P026002-2, Dako, Denmark) were used. They were diluted to 1:5000 in TBS with 3% BSA and incubated for 1 hour at room temperature under agitation. Eventually, after washing, the HRP activity was revealed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher, USA) and detected in a Chemi Doc MP imaging system (BioRad, USA). WB results were analyzed by measuring the mean gray value of protein bands delimited in ROIs using ImageJ software (Rasband WS, USA). The relative expression of P-gp to β -Actin and PXR to Histone H3 was calculated for each sample, and the average of samples for each group was normalized to the control.

2.11. Statistical analysis

Descriptive data are presented as mean $\pm$ standard deviation (SD) whereas the results of the statistical analysis are shown as mean $\pm$ SE unless otherwise indicated. IBM SPSS 23 was used for the statistical analysis. Generalized Estimated Equation (GEE) analysis with the independent matrix (34) was used to assess the differences in the SUV-TAC of the blood and brain. Generalized Linear Models (GLM) were used to evaluate the differences in SUV_{40-60min}, pharmacokinetic parameters (V_T , K_1 , k_2), and blood kinetics (K_e) among the groups, independently for each region. P values < 0.05 were considered statistically significant. Relative changes among the groups were calculated, using the control as the reference.

3. Results

3.1. Tracer production

The final product was obtained in $5.6 \pm 2.9\%$ yield (Mean $\pm$ SD) (n=9) (decay-corrected) in an average synthesis time of 121 ± 24 min (Mean $\pm$ SD). The radiochemical purity of [¹⁸F]MC225 was $95.3 \pm 2.7\%$ (Mean $\pm$ SD) and the molar activity higher than 80 TBq/mmol (Mean $\pm$ SD).

3.2. Plasma kinetics and metabolism of [¹⁸F]MC225

No significant differences between the control and the treated animals were found concerning the parent fraction in plasma. However, statistical analysis revealed that the (metabolite-corrected) SUV-TAC of [¹⁸F]MC225 in plasma was higher in the high-dose group than in the other two groups. The SUV-TAC of the tracer was 44% ($p = 0.042$) higher in the high-dose group than in the control group (high-dose group = 0.97 ± 0.10 , low-dose group = 0.67 ± 0.06 , control group = 0.70 ± 0.10) (Figure 2).

Figure 2: Metabolite-corrected SUV-TACs for plasma of the control (blue), low-dose (green), and high-dose group (red)(A) and the percentage of plasma radioactivity representing [^{18}F]MC225 as a function of time (B).

The statistical analysis found a significant increase in the K_e in the 6 mg/kg treated group. The K_e from the high-dose group increased by 25% compared to the control group (K_e high-dose group = $0.048 \pm 0.003 \text{ seconds}^{-1}$ vs K_e control group = $0.039 \pm 0.003 \text{ seconds}^{-1}$, $p = 0.014$). However, no significant differences were found between the control group and the low-dose

group (Ke low-dose group = 0.035 ± 0.003 seconds $^{-1}$ vs Ke control group = 0.039 ± 0.002 seconds $^{-1}$, $p = 0.35$).

3.3. Brain kinetics of [^{18}F]MC225

3.3.1. The SUV-TAC of the whole brain

SUV-TACs for the whole brain were calculated. In all groups, [^{18}F]MC225 showed an initially high uptake followed by a subsequent decrease (Figure 3). In all groups, the peak was observed at 3.3 ± 2.4 minutes (mean $\pm$ SD) after injection of the tracer. Statistical analysis did not find any significant differences in the SUV-TAC of the whole brain among the groups.

Figure 3: SUV TACs for whole brain in the control (blue color), low-dose (4.5 mg/kg) (green color), and high-dose group (6 mg/kg) (red color).

3.3.2. $\text{SUV}_{40-60\text{min}}$ of the whole-brain region

Since the last part of the PET scans shows more stable SUV values, a mean SUV value from 40 to 60 min was calculated for all the animals ($\text{SUV}_{40-60\text{ min}}$). The $\text{SUV}_{40-60\text{ min}}$ calculated using the last 20 minutes of the scan decreased in the high-dose group with regard to the control group but this difference was not statistically significant ($\text{SUV}_{40-60\text{ min}}$ control = 0.45 ± 0.02 ; $\text{SUV}_{40-60\text{ min}}$ low-dose = 0.45 ± 0.02 ; $\text{SUV}_{40-60\text{ min}}$ high-dose = 0.40 ± 0.02).

3.3.3. Pharmacokinetic Modelling

A dose of 4.5 mg/kg MC111 decreased the V_T and K_1 values compared to the control group, however, the decrease was not statistically significant. The higher dose (6 mg/kg) caused a significant reduction in the K_1 and V_T values in all the brain regions compared to the control group. The analysis also found significant differences in K_1 and V_T between the high-dose group and the low-dose group. Figure 4 shows the V_T , K_1 , and k_2 values of all the brain regions in all the experimental groups.

Figure 4: Mean $\pm$ SE values of K_1 (A), V_T (B) and k_2 (C) in the control (blue), low-dose (4.5 mg/kg) (green) and high-dose (6 mg/kg) (red) groups.

The whole-brain region K_1 significantly decreased by 34% ($p < 0.001$) after the high-dose treatment compared to the control group, and the whole-brain region V_T also decreased by 24% ($p < 0.001$). A similar trend was observed in all brain regions. The largest change in the K_1 was observed in the septum, the superior colliculus, and the cortex region with a decrease of 39%, 38%, and 36% compared to the control group, respectively. In the case of the V_T , the largest changes were found in the brainstem, the thalamus, and the superior colliculus with reductions of 30%, 27%, and 26% respectively (more information in table 1). The analysis did not find significant differences in k_2 values between the low-dose group and the control in any of the brain regions. However, k_2 values decreased in the high-dose group compared to control.

Table 3: Mean $\pm$ SE K_1 and V_T values of all selected brain regions in all experimental groups, percentages of change relative to the control group, and p-values.

Region	Group	K_1 (mL/mL/min)			V_T (mL/mL)		
		Mean $\pm$ SE	Changes relative to Control (%)	p-value	Mean $\pm$ SE	Changes relative to Control (%)	p-value
Amygdala	Control	0.27 $\pm$ 0.02			7.38 $\pm$ 0.47		
	4.5mg/kg	0.27 $\pm$ 0.02	-0.94	0.92	6.94 $\pm$ 0.47	-6.03	0.50
	6mg/kg	0.19 $\pm$ 0.02	-30.62	0.00	5.41 $\pm$ 0.51	-26.81	0.00
Basal Ganglia	Control	0.27 $\pm$ 0.02			6.23 $\pm$ 0.33		
	4.5mg/kg	0.26 $\pm$ 0.02	-4.63	0.62	5.89 $\pm$ 0.33	-5.53	0.46
	6mg/kg	0.19 $\pm$ 0.02	-30.43	0.00	4.6 $\pm$ 0.36	-26.19	0.00
Brainstem	Control	0.41 $\pm$ 0.03			8.66 $\pm$ 0.43		
	4.5mg/kg	0.37 $\pm$ 0.03	-8.80	0.31	7.88 $\pm$ 0.43	-9.02	0.20
	6mg/kg	0.29 $\pm$ 0.03	-29.33	0.00	6.1 $\pm$ 0.48	-29.63	0.00
Cerebellum	Control	0.38 $\pm$ 0.02			8.7 $\pm$ 0.49		
	4.5mg/kg	0.36 $\pm$ 0.02	-4.77	0.58	8.18 $\pm$ 0.49	-6.01	0.45
	6mg/kg	0.26 $\pm$ 0.03	-30.60	0.00	6.41 $\pm$ 0.54	-26.37	0.00
Corpus Callosum	Control	0.26 $\pm$ 0.02			7.25 $\pm$ 0.34		
	4.5mg/kg	0.23 $\pm$ 0.02	-8.00	0.43	7.04 $\pm$ 0.34	-2.80	0.68
	6mg/kg	0.17 $\pm$ 0.02	-34.47	0.00	5.65 $\pm$ 0.37	-22.08	0.00
Cortex	Control	0.27 $\pm$ 0.02			8.57 $\pm$ 0.41		
	4.5mg/kg	0.24 $\pm$ 0.02	-9.65	0.29	8.07 $\pm$ 0.41	-5.89	0.38
	6mg/kg	0.17 $\pm$ 0.02	-36.39	0.00	6.76 $\pm$ 0.45	-21.19	0.00
Hippocampus	Control	0.27 $\pm$ 0.02			6.8 $\pm$ 0.35		
	4.5mg/kg	0.26 $\pm$ 0.02	-1.33	0.90	6.5 $\pm$ 0.35	-4.48	0.54
	6mg/kg	0.18 $\pm$ 0.02	-33.77	0.00	5.24 $\pm$ 0.38	-22.94	0.00
Hypothalamus	Control	0.35 $\pm$ 0.03			7.57 $\pm$ 0.37		
	4.5mg/kg	0.33 $\pm$ 0.03	-3.30	0.75	7.27 $\pm$ 0.37	-3.96	0.57
	6mg/kg	0.24 $\pm$ 0.03	-30.33	0.00	6.08 $\pm$ 0.41	-19.61	0.01
Medial Geniculate	Control	0.32 $\pm$ 0.03			6.06 $\pm$ 0.29		
	4.5mg/kg	0.3 $\pm$ 0.03	-5.13	0.72	5.85 $\pm$ 0.29	-3.59	0.59

	6mg/kg	0.2 ± 0.04	-35.96	0.02	4.65 ± 0.32	-23.34	0.00
Mesencephalic Region	Control	0.35 ± 0.02			7.14 ± 0.33		
	4.5mg/kg	0.34 ± 0.02	-1.86	0.83	6.65 ± 0.33	-6.75	0.31
	6mg/kg	0.26 ± 0.02	-26.91	0.00	5.49 ± 0.37	-23.00	0.00
MidBrain	Control	0.35 ± 0.02			7.07 ± 0.34		
	4.5mg/kg	0.33 ± 0.02	-5.43	0.52	6.92 ± 0.34	-2.13	0.75
	6mg/kg	0.26 ± 0.02	-25.27	0.00	5.58 ± 0.37	-21.06	0.00
Septum	Control	0.27 ± 0.02			6.37 ± 0.35		
	4.5mg/kg	0.26 ± 0.02	-5.95	0.60	5.91 ± 0.35	-7.23	0.35
	6mg/kg	0.17 ± 0.02	-38.90	0.00	4.89 ± 0.38	-23.28	0.00
Striatum	Control	0.26 ± 0.02			6.36 ± 0.32		
	4.5mg/kg	0.24 ± 0.02	-9.46	0.33	6.05 ± 0.32	-5.00	0.48
	6mg/kg	0.17 ± 0.02	-35.30	0.00	4.95 ± 0.35	-22.18	0.00
Superior Colliculus	Control	0.39 ± 0.03			6.98 ± 0.33		
	4.5mg/kg	0.36 ± 0.03	-9.66	0.30	6.34 ± 0.33	-9.21	0.17
	6mg/kg	0.25 ± 0.03	-37.67	0.00	5.13 ± 0.36	-26.42	0.00
Thalamus	Control	0.3 ± 0.02			6.19 ± 0.31		
	4.5mg/kg	0.29 ± 0.02	-3.23	0.76	5.89 ± 0.31	-4.82	0.49
	6mg/kg	0.2 ± 0.03	-33.61	0.00	4.54 ± 0.34	-26.70	0.00
Whole-Brain	Control	0.3 ± 0.02			7.82 ± 0.38		
	4.5mg/kg	0.28 ± 0.02	-7.18	0.42	7.35 ± 0.38	-5.99	0.38
	6mg/kg	0.2 ± 0.02	-33.68	0.00	5.92 ± 0.41	-24.29	0.00

3.3.4. Parametric images

Figure 5 shows parametric maps of K_1 , V_T , and k_2 in three representative subjects of each group using 1-TCM.

Figure 5: Parametric images calculated using 1-TCM and 60-min scan duration of three representative subjects of each group: control, low-dose (4.5 mg/kg), and high-dose (6 mg/kg).

3.4. Western blot

Western blot analysis showed no statistical differences between the control and treated groups. There was a noticeable intragroup variability regarding P-gp analysis, but no significant differences were found (Figure 6). In PXR analysis, there was a tendency to lower levels of PXR in the high dose group (Figure 7).

Figure 6: P-gp expression levels and western blot bands corresponding to P-gp (band close to 160 kDa predicted molecular weight) and β -actin as load control (band close to 42 kDa predicted molecular weight).

Figure 7: PXR expression levels and western blot bands corresponding to PXR (two bands close to 50 kDa predicted molecular weight) and Histone H3 as load control (band close to 17 kDa predicted molecular weight). Notice that in our analysis only the 50 kDa band was quantified.

4. Discussion

The present study was designed to determine the effect of MC111 on the P-gp function *in vivo* using the P-gp tracer [^{18}F]MC225 and PET. Our analysis highlights the induction effect of MC111 on the P-gp function at the BBB of healthy rats. This finding was in line with the results that were previously observed *in vitro* (20). Moreover, the results demonstrate that [^{18}F]MC225 can be used to measure increases in the P-gp function reflected in decreases in K_1 and V_T values of [^{18}F]MC225 in all brain regions. Western blot analyses were also performed using the whole brain of the rats; however, the results did not find changes in the P-gp expression in rats treated with the inducer.

The blood analysis revealed that the treatment with MC111 did not alter the metabolism of the tracer since the analysis did not find significant differences in the percentages of radio-metabolites among the groups. However, the treatment with 6 mg/kg dose of MC111 increased the (metabolite-corrected) concentration of the tracer in plasma compared to the control group and low-dose group. However, the elimination of the tracer from plasma (K_e) was also higher in the high-dose group than in the other two groups, which means that the washout of the tracer from the blood was faster in the high-dose group. Therefore, it seems that although the concentration of the tracer in plasma was higher in the high-dose group, the washout of the tracer was also faster. One rat (from the high-dose group) showed very high concentration levels of [^{18}F]MC225 in blood reaching SUV values of 6 in blood and plasma. Some unexpected event must have occurred during the treatment and scan of this animal since all the analyses were carried out following the same procedures as were used in the other animals. Due to the unrealistically high SUV values in blood, this animal was excluded from the analysis.

Analysis of the whole-brain SUV-TACs did not find significant differences among the experimental groups. The SUV calculated using the last 20 minutes of the scan showed a reduction of 10% in the high-dose group compared to control, however, this reduction in the $\text{SUV}_{40-60\text{min}}$ was not statistically significant. These results refute the use of SUV for quantification of P-gp induction and support the use of pharmacokinetic parameters.

Following previous recommendations, the kinetics of [^{18}F]MC225 were analyzed using 1-TCM (24). The statistical analysis found a significant decrease in the K_1 and V_T values in animals treated with high-dose of MC111 compared to the control and low-dose groups, which indicates an increase in the P-gp function. The whole-brain region K_1 in the high-dose group decreased by 34% ($p < 0.001$) compared to the control group and by 29% ($p = 0.005$) compared to the low-dose group. The V_T of the whole-brain region in the high-dose group decreased by 24% ($p = 0.001$) compared to control and by 19% ($p = 0.011$) compared to the low-dose group. Similar changes were observed in all the selected brain regions. As was also observed in previous studies, changes in K_1 are larger than the change in V_T . For this reason, K_1 has been identified as a more sensitive parameter to measure the P-gp function at the BBB in rats and in non-human primates (24,35), however, V_T can also be used as a surrogate parameter to estimate the P-gp function (24).

Many therapeutic drugs have shown an induction effect on the P-gp transporter (36). Subchronic morphine administrations induced P-gp expression in brain microvessels of rats (37). In the mentioned study, rats were treated with increasing doses of morphine by intraperitoneal injections (i.p.) twice a day for 5 consecutive days. Six hours after the last dose of morphine, the levels of P-gp were not higher but after 24 hours of abstinence, the P-gp expression increased 1.5-times compared to control animals (37). Long-term exposure (60-days) to phenobarbital, phenytoin, carbamazepine, and valproic acid (antiepileptic drugs) increased the P-gp expression levels and

P-gp function in rat brain microvascular endothelial cells *in vitro* (38). P-gp expression in brain capillaries of mice was also increased 24 hours after the administration of rifampin (50 mg/kg). That study also observed an increase of P-gp expression and function *in vitro* using isolated brain capillaries after the exposure to rifampicin and hyperforin, two PXR-ligands (39). After the treatment with the corticosteroid dexamethasone for 4 days at dose 25 mg/kg i.p., brain capillaries of rats showed an increase in the P-gp expression (40). The previously described studies evaluated the P-gp function *in vitro* using endothelial cells acquired from brain post mortem. However, our study confirms the P-gp induction effect of MC111 *in vivo* using PET imaging. This compound has been selected from an extensive P-gp/BCRP library and *in vitro* studies have already shown that MC111 increases the P-gp and BCRP function and expression (20). Several studies have suggested a synergetic role between the two efflux transporters (10). Moreover, the expression of these two transporters is usually increased in various neurodegenerative diseases (22). Therefore, it is expected that the dual action of MC111 may be beneficial for the treatment of AD.

Since P-gp function can be altered after several treatments, it is important to monitor such alterations to prevent decreases in drug efficacy or drug toxicities. Moreover, since P-gp inducers have the ability to restore the P-gp function and thus can be a potential treatment for neurodegenerative diseases where the P-gp function is decreased, PET imaging with P-gp tracers may allow the evaluation of the effect of promising inducer drug candidates.

Tracers for imaging P-gp function such as [¹¹C]verapamil and [¹¹C]-N-desmethyl-loperamide show very low uptake inside the brain at baseline condition due to their strong affinity toward the transporter protein, thus hampering the evaluation of P-gp induction (41–44). However, weak P-gp substrates such as [¹⁸F]MC225 show higher baseline brain uptake levels than [¹¹C]verapamil (24) and thus have been proposed as adequate tracers to measure P-gp induction. The results of our studies confirm the ability of [¹⁸F]MC225 to measure increases in the P-gp function because the treatment with the P-gp inducer MC111 significantly decreased the K_i and V_T values of [¹⁸F]MC225 in the high-dose group compared to control group, indicating an increase in the P-gp function in these rats.

Increases in the P-gp function do not always mean increases in the P-gp expression, for this reason, it is important to assess simultaneously the P-gp expression and function to identify the mechanism of action of a drug. For instance, western blot and flow cytometry analysis indicated that colchicine increased the P-gp expression in caco-2 cells without increasing the P-gp function (45). In the present study, the P-gp expression was assessed postmortem using the whole-brain of the rats. The results did not indicate any changes in the P-gp expression among the groups. Thus, the observed increase of P-gp function after administration of 6 mg/kg MC111 may be caused by relocation of intracellular P-gp protein molecules to the luminal plasma membrane of endothelial cells (10,11). However, to adequately evaluate the P-gp expression at the BBB of rats and to not

overestimate the P-gp levels, the brain endothelial cells must be isolated because the P-gp transporter is also expressed at low levels by non-endothelial cell types and because the volume of the brain capillaries represents approximately 1% of the total brain volume (46–48). Moreover, P-gp transporters are also expressed in intracellular compartments whereas only the transporter molecules on the cell surface are functional (49,50). Therefore, it is important to use antibodies that specifically recognize an external epitope of the P-gp transporter to avoid the detection of P-gp stored in intracellular vesicles (46,51,52). Therefore, future studies will be carried out to assess the P-gp expression levels in isolated brain endothelial cells to obtain a more accurate quantification. Our study also evaluated the PXR expression level in the whole brain tissue, since we have hypothesized that MC111 may increase the P-gp expression by the activation of the PXR. However, the analysis did not detect any increase in the expression levels of this nuclear receptor. Interestingly, in the PXR western blot, a band between 37 and 50 kDa was also found in addition to the expected PXR 50 kDa band. This could be due to the presence of alternative splicing variants. Isoforms 1b and 2b of 43 and 39 kDa respectively were reported in uniprot database but further analysis is needed in order to elucidate this issue (53). Notice that in our analysis only the 50 kDa band quantification is shown.

5. Conclusion

In conclusion, this study shows the ability of MC111 to induce the P-gp function at the BBB of rats by PET imaging using the P-gp PET tracer [^{18}F]MC225. Postmortem western blot analysis did not detect increases in the P-gp expression in treated rats, however, changes in the P-gp function did not always align with changes in the P-gp expression. Although changes in the P-gp expression were not detected, this study demonstrated that the administration of a 6 mg/kg dose of MC111 decreased the K_i and V_T values of [^{18}F]MC225, which indicates an increase of the P-gp function. Thereby, the results also verify the ability of [^{18}F]MC225 to measure increases in the P-gp function of rats. [^{18}F]MC225 may be the first radiofluorinated tracer able to monitor increases and decreases of the P-gp function at the mammalian BBB. Moreover, this technique may be used to screen potential P-gp inducers that can be applied as experimental treatments for several neurodegenerative diseases.

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Chapter 9

General Discussion, Future Perspectives, and Concluding Remarks

General Discussion

Previous studies have already shown that [^{18}F]MC225 is a good radiotracer for measuring decreases in the P-glycoprotein (P-gp) function at the blood-brain barrier (BBB) of rodents using Positron Emission Tomography (PET) (1,2). However, other characteristics of the tracer should be closely examined before its clinical application. For instance, the reproducibility of [^{18}F]MC225 PET scans must be tested before the performance of longitudinal studies, and the characteristics of [^{18}F]MC225 can be validated in non-human primates as P-gp expression is more similar to humans in contrast to rodents. Additionally, the ability of the tracer to detect increases in the P-gp function needs to be confirmed. Therefore, the main aim of this thesis is to evaluate the pharmacokinetics of the P-gp tracer [^{18}F]MC225 using PET scans under different conditions and in several species.

The purpose of this chapter is to discuss the most important findings of the previously described chapters and suggest future research on the topic.

Pharmacokinetic evaluation of [^{18}F]MC225

The delivery of many drugs to the Central Nervous System (CNS) is reduced by the action of efflux transporters in the BBB, particularly by the P-gp transporter. This transporter is known to limit the brain entry of antipsychotics, antidepressants, anti-epileptic drugs, chemotherapeutic agents (3), and a large number of new CNS drug candidates (4). Moreover, many compounds can interact with the P-gp transporter and cause changes in its function and/or expression, thus, the P-gp transporter is involved in several drug-drug interactions (DDIs) at the BBB (5,6). Additionally, altered P-gp function has been found in several brain disorders (7). A decline in its function has been observed in Alzheimer's disease patients (8,9) and an increase in patients with intractable epilepsy (10). Since many factors such as diet, stress, and medication can alter the function or expression of this transporter (6), it is of interest to monitor the P-gp function *in vivo* using PET imaging. The performance of longitudinal PET studies may allow the monitoring of the P-gp function at different time points and [^{18}F]MC225 PET could contribute to elucidate the role of this transporter in the onset or development of several brain pathologies.

However, before performing longitudinal studies, the reproducibility of [^{18}F]MC225 PET scans needs to be assessed, since many factors including diet, gender, and anesthesia can influence the pharmacokinetic outcomes (11,12). Moreover, studies in small animals require the use of anesthesia to immobilize the animals during the acquisition of the scan, thus, in longitudinal PET studies, animals are repeated anesthetized. **Chapter 3** evaluates the reproducibility of [^{18}F]MC225 PET and the effect of repeated exposures to anesthesia on the P-gp function. The

results showed an adequate reproducibility of the [^{18}F]MC225 influx constant K_1 values. The study concluded that [^{18}F]MC225 longitudinal PET scans can be performed to monitor P-gp function at different time points during disease progression or after drug treatment. However, the reproducibility of the PET scans needs to be re-assessed if the experimental conditions and procedures change as the use of female rats or different strains or species. Moreover, this study only investigated the effect of isoflurane-anesthesia. To ensure an adequate reproducibility, the effect of another type of anesthetic on the P-gp function at the BBB should be evaluated before conducting longitudinal studies. Additionally, it has been described that the P-gp function has a daily rhythm and displays a reduced function at the beginning of the active phase (dark phase) (13). For this reason, the studies of **Chapter 3** were carried out during the light phase of the animals, between the zeitgeber time 3 (ZT, 3 hours after light onset) and ZT9 (9 hours after light onset). We recommend performing the longitudinal studies always at the same hour to avoid erroneous PET quantification due to the daily fluctuations of the P-gp function.

The main aim is to translate the [^{18}F]MC225 to the clinical settings, therefore, the kinetics of the tracer should be evaluated in a species more closely related to humans than rodents. Several studies have found that P-gp function and expression in rodents is higher than in humans, meanwhile, the expression and function in non-human primates is more similar (14,15). For this reason, the pharmacokinetics of [^{18}F]MC225 were assessed in non-human primates before and after the inhibition of the P-gp function. This kinetic evaluation described in **Chapter 4** found large standard errors (SE%) in the estimated V_T and k_2 parameters using the 2-Tissue Compartment model (TCM) and short scan durations. For this reason, the 1-TCM was selected as the preferred model for the analysis of short scan durations (10- to 30-min) and the 2-TCM for the long scan durations (60-and 91-min). Similar unrealistically high SE% of V_T were found in the kinetic analysis of [^{18}F]MC225 performed in rats when the 2-TCM was used to fit the data (2). Regarding the effect of the P-gp inhibition, similar to the results obtained in rats, the K_1 and V_T values of all the brain regions increased after the injection of the P-gp inhibitor. The K_1 and V_T values at baseline were comparable to those obtained in rats, however, after the P-gp inhibition, the values were larger in rats than in non-human primates (2,16). This difference may be explained by the dose of tariquidar used in these experiments. Meanwhile, 8 mg/kg of tariquidar is considered the maximum effective dose in rodents (2,17), in non-human primates, this dose may not cause complete inhibition. The study concluded that the P-gp function at the BBB in non-human primates can be measured with [^{18}F]MC225 using either the K_1 obtained by applying 1-TCM to short scan durations or using K_1 and V_T obtained with 2-TCM and long scan durations.

Next, the pharmacokinetics of strong P-gp substrate, the (*R*)-[^{11}C]verapamil, were also evaluated in non-human primates (**Chapter 5**). The pharmacokinetic evaluation of [^{18}F]MC225 and (*R*)-

[^{11}C]verapamil in the same species would allow the head-to-head comparison of both tracers in a species more closely related to humans than rodents in terms of P-gp expression (14) (**Chapter 6**). The results of **Chapter 5** found that the model of choice to fit the (*R*)-[^{11}C]verapamil data either at baseline or after the P-gp inhibition was the 1-TCM regardless of the scan duration. In this case, the kinetic parameter most affected by the P-gp inhibition was the V_T instead of the K_1 as observed in the [^{18}F]MC225 kinetic analysis. Regarding other species, the V_T values of (*R*)-[^{11}C]verapamil were larger than those obtained in rats and humans, only baseline K_1 values in rats were similar to those obtained in non-human primates (17–20). Although (*R*)-[^{11}C]verapamil has been used to measure the P-gp function before and after the P-gp inhibition in humans and rats, we could not compare the results obtained in non-human primates with those obtained in humans and rats due to the differences in the study protocols (the use of different P-gp inhibitors, dose administrated, duration of the PET scan) (18,19,21). The study recommends the use of 1-TCM and K_1 to measure the P-gp function at the BBB of non-human primates with (*R*)-[^{11}C]verapamil.

One important limitation of the pharmacokinetic studies previously described is that those studies did not account for the lipophilic radio-metabolites in plasma. Lipophilic radio-metabolites can cross the BBB by passive diffusion and accumulate inside the brain, thus they can contribute to the radioactive signal detected in the brain and lead to erroneous kinetic quantifications. The metabolite analysis of these studies only focused on the polar radio-metabolites; thus, the amount of lipophilic radio-metabolites formed during the metabolism of the tracers is unknown. Moreover, the concentration of radiotracer in plasma corrected for radio-metabolites could be overestimated because only polar radio-metabolites are taken into account in the correction. This fact is especially important in the case of (*R*)-[^{11}C]verapamil since it is already known that the metabolism of this tracer in rats and humans leads to the formation of lipophilic radio-metabolites (22–24). In the case of [^{18}F]MC225 a more extensive metabolite analysis was performed in rats and the results showed that the majority of radio-metabolites were more polar than the parent fraction (2). To avoid erroneous PET quantifications, all previous studies suggest the use of short scan durations to limit the effect of the metabolism of the tracer in the pharmacokinetic analysis. Moreover, short scans are also preferred in the clinical setting to reduce the scanning time of the patient. This can be especially important for patients with neurodegenerative diseases as Alzheimer's or Parkinson's. For those patients to remain immobile during the PET scan can be difficult and can cause high levels of stress.

[^{18}F]MC225 is considered as a weak P-gp substrate which results in a higher baseline uptake under normal conditions which may facilitate the detection of increases in the P-gp function. In fact, previous preclinical studies in rats have already detected higher baseline V_T and SUV values of [^{18}F]MC225 compared to (*R*)-[^{11}C]verapamil. To confirm this behavior in non-human primates, a head-to-head comparison with the strong P-gp substrate tracer (*R*)-[^{11}C]verapamil was

performed. As it was expected, the results of **Chapter 6** showed that the baseline V_T values of [^{18}F]MC225 were higher than those of (*R*)-[^{11}C]verapamil due to its less avidity for the transporter. However, baseline K_1 values of both tracers were not significantly different. With regards to the after-inhibition scans, K_1 and k_2 values of (*R*)-[^{11}C]verapamil were significantly higher than those of [^{18}F]MC225, whereas V_T values of both tracers after P-gp inhibition were not significantly different. It was expected that the administration of the same dose of the P-gp inhibitor would allow the entrance of both tracers inside the brain and reach similar K_1 and V_T values. However, the highest K_1 after-inhibition values of (*R*)-[^{11}C]verapamil suggests that this parameter is affected by other non-specific signals. In contrast to the recommendations of **Chapter 5**, these findings suggest that K_1 of (*R*)-[^{11}C]verapamil may not be the most adequate parameter to measure the P-gp function. In addition, this head-to-head comparison revealed significant regional differences in K_1 , V_T , and k_2 distributions for both tracers at baseline which disappeared after the P-gp inhibition. However, the highest baseline V_T and K_1 values for both tracers were found in the same regions (occipital, orbitofrontal cortex, and cerebellum) as in previous analysis in rats (2,17). These higher values in these regions at baseline suggest that cerebellum and cortical regions may display a lower P-gp function compared to other brain regions. **Chapter 6** concludes that the 30-min V_T values of both tracers show comparable sensitivity to measure decreases in the P-gp function in non-human primates. Furthermore, the results suggest that the higher baseline V_T of [^{18}F]MC225 may allow the quantification of increases in the P-gp function, however, further studies need to be carried out to validate this hypothesis.

Figure 1 shows the difference in the affinity towards the P-gp transporter of [^{18}F]MC225 and (*R*)-[^{11}C]verapamil. The lower affinity of [^{18}F]MC225 leads to a higher V_T inside the brain at baseline conditions ([^{18}F]MC225 $V_T=6.05\pm0.45$ and (*R*)-[^{11}C]verapamil $V_T=2.52\pm0.32$). After the injection of the same dose of tariquidar, both tracers reached similar V_T values ([^{18}F]MC225 $V_T=8.04\pm0.24$ and (*R*)-[^{11}C]verapamil $V_T=8.87\pm0.20$).

Figure 1: Parametric images showing the V_T of [¹⁸F]MC225 and (R)-[¹¹C]verapamil inside the brain. The [¹⁸F]MC225 shows higher baseline V_T due to the lower affinity toward the P-gp transporter compared to (R)-[¹¹C]verapamil.

[¹⁸F]MC225 measures increases in the P-gp function

Previous studies have already suggested the potential ability of [¹⁸F]MC225 to measure increases in the P-gp function. Finally, **Chapter 8** confirms this characteristic. The administration of a P-gp inducer (MC111) caused a decrease in the K_1 and V_T values of [¹⁸F]MC225, indicating an increase in the P-gp function at the BBB of treated rats. However, the western blot experiments performed ex vivo did not find any increase in the P-gp expression in the treated groups. One important limitation of our study is that western blot experiments were done using the whole brain tissue instead of isolated brain endothelial cells as it is usually done (25). The P-gp is expressed in most of the cells at low levels and the volume of the capillary endothelium occupies less than 1% of the total brain volume, thus, it is needed to isolate the brain endothelial cells to adequately measure the P-gp expression at the BBB (26–28). Moreover, the P-gp transporter may also be expressed in the internal part of the cells. Thus, it is important to distinguish the transporter expressed at the intracellular part from the one expressed at the cell surface. The latter being the one able to exert the efflux function (26,29,30). For this reason, it is important to use antibodies that specifically recognize an external epitope of the P-gp transporter (31,32). To clarify if the P-gp inducer, MC111, is able to increase the P-gp expression at the brain endothelial cells in rats, additional studies in isolated brain capillaries are currently ongoing. Nevertheless, many mechanisms can regulate the expression of this transporter (33–35). The results of this chapter suggest that the increase in the P-gp function after the administration of MC111 may be caused by the relocation of intracellular P-gp protein molecules to the luminal plasma membrane of endothelial cells (33,34). **Chapter 8** concluded that the administration of MC111 decreased K_1 and V_T values of [¹⁸F]MC225 in all the brain regions which indicates an increase in the P-gp

function. Moreover, the results also confirm the ability of [^{18}F]MC225 to measure increases in the P-gp function at the BBB of rats. The chapter indicates that [^{18}F]MC225 has the potential to become the first radiofluorinated tracer able to monitor increases and decreases of the P-gp function at the BBB.

The kinetic evaluation of [^{18}F]MC225 under P-gp inhibition and P-gp induction conditions revealed that the parameter most affected by the challenges was the influx constant K_1 . Thereby, these studies confirmed that the best parameter to measure the P-gp function at the BBB with the [^{18}F]MC225 tracer may be the K_1 . This parameter depends on blood flow (F) and the unidirectional extraction fraction (E_u) (36,37) ($K_1 = F * E_u$) and since [^{18}F]MC225 is a lipophilic tracer ($\log D=3.0$) (1), it has a large extraction fraction and therefore K_1 is more susceptible to changes in the blood flow (38). For this reason, it has been recommended the performance of an additional PET study (e.g. using [^{15}O]H $_2$ O) for measuring the blood flow to assess the P-gp function more precisely (39,40). However, if inhibition or induction of P-gp caused changes in the blood flow, the k_2 values of the tracer would have also increased (after the P-gp inhibition) or decreased (after the P-gp induction) because this parameter also possesses information about blood flow (41) ($k_2 = K_1/V_T$). Previous studies did not find any significant change in the k_2 values, thus K_1 values may not be affected by changes in the blood flow in our studies. Nevertheless, future studies in patients can include a dual-protocol where, first, the blood flow is studied with an [^{15}O]H $_2$ O PET scan, and subsequently, the P-gp function is evaluated with [^{18}F]MC225.

Additionally, these studies have shown that the regions most affected by the challenges were the subcortical regions. The regions most affected by the P-gp inhibition in rats were the hippocampus and striatum (2) and in non-human primates, it was the striatum and thalamus (16). With regards to the P-gp induction, the largest changes in K_1 have been observed in the septum and superior colliculus. Therefore, it was suggested that subcortical regions may present a higher P-gp function than the frontal cortex and cerebellum, however, further studies are needed to verify these findings.

Translation of [^{18}F]MC225 to the clinical settings

The evaluation of [^{18}F]MC225 in rodents and non-human primates supports the translation of the tracer to the clinical settings. However, before translation to the clinic, preclinical toxicology and dosimetry studies were performed to investigate the safety profile of the tracer. The studies showed that the potential toxicological effect of [^{18}F]MC225 was in an acceptable range and the estimated effective dose was within the limits established by the International Commission on Radiological Protection (42).

Moreover, prior to the administration of the tracer to humans, the production of [^{18}F]MC225 must be performed following the current guidelines of Good Manufacturing Practice (GMP) (43,44). The radiosynthesis of [^{18}F]MC225 involves two steps including the distillation of [^{18}F]fluoroethylbromide intermediate (1). To make the reaction simpler, faster, and more robust, it was of interest to simplify the synthesis route. This shortened production of the tracer would be easier to automate and may facilitate the translation of [^{18}F]MC225 to the clinical. In **Chapter 7**, a new MC225 precursor compound that allows the production of the radiotracer by direct ^{18}F -fluorination was synthesized. To this end, a mesylate MC225 precursor salt was synthesized, however, the final compound shows various impurities. Several methods were used to further purify the compound, but the results were unsatisfactory. Thus, this synthesis could not be used to produce [^{18}F]MC225 for clinical uses, since the GMP guidelines require the use of highly pure starting material. Nevertheless, the mesylate precursor was used in the radiolabeling procedure, the one-step synthesis was automated in the IBA Synthera module, and [^{18}F]MC225 was successfully produced. Further studies are required to obtain a suitable purification method of the mesylate precursor and thus, obtain a GMP compliant synthesis. Nowadays, the conventional two-step synthesis has been successfully automated and it is still the procedure used for the production of [^{18}F]MC225 for clinical trials (42).

Summarizing the results of the chapters in this thesis together with the positive results obtained in the mutagenicity test, toxicology, and dosimetry studies as well as the successful automation of the 2-step synthesis (42), it can be concluded that [^{18}F]MC225 is suitable for performing clinical PET studies.

Future Perspectives

The therapeutic effect of P-gp modulators

The first P-gp modulators were used to overcome drug resistance, particularly, in cancer. Many tumors show an increased function and expression of the P-gp transporter that impede the delivery of chemotherapeutic agents to the target tissue, which decreases the treatment efficacy (45,46). Thus, P-gp inhibitors were used in combination with chemotherapeutic drugs to improve the effectiveness of therapies. However, the clinical trials did not show positive results with the currently available P-gp inhibitors, and the studies were stopped due to toxicity side-effects (6). Nowadays, a next generation of P-gp inhibitors has been proposed which include compounds extracted from natural products such as flavonoids, alkaloids, or coumarins as well as surfactants as tween-20 (6). Also, recently xanthenes derivatives and 5-oxo-hexahydroquinoline derivatives have been proposed as improved P-gp inhibitors with dual biological activity since they are able to efficiently inhibit the P-gp activity and the cell growth or be able to simultaneously blocking the

activity of several ABC transporters (47,48). Apart from the use of hybrid molecules able to interact with different targets within the tumor tissue, other strategies have been proposed to overcome drug resistances such as the use of nanocarrier-mediated transport, which would guide the therapy to a specific target. The combination of the anticancer drug and the P-gp inhibitor in a nanoparticle would increase the permeability of the treatment due to the inhibition of the P-gp function and allow the controlled release of the anticancer drug in the target tissue (49–52). This increase in the selectivity and specificity may allow the local inhibition of the P-gp function and the increase of the bioavailability of the drug only in the desired cells, avoiding unexpected toxicities. Patients with intractable epilepsy and treatment-resistant depression have shown an increased P-gp function which also limits the effectiveness of the treatments (10,53–55). These novel P-gp inhibitors may also help to overcome drug resistance in these diseases.

On the other hand, P-gp inducers have been proposed as potential treatments to restore the P-gp function in several brain disorders where the P-gp function is decreased such as Alzheimer's disease (7,56–58). These compounds act by increasing the protein expression which can be induced by a wide variety of mechanisms (6). P-gp inducer may promote the efflux of amyloid- β (A β) (a P-gp substrate (59)), outside the brain which its accumulation is considered as the main hallmark of this disease (60,61). Moreover, preclinical studies in Alzheimer's disease models have found that the administration of a P-gp inducer not only reduced the A β accumulation inside the brain but also improved the cognitive function of these animals (62,63). P-gp inducers as the one described in **Chapter 8** may increase the P-gp function, restore the clearance of the A β and improve the cognitive symptoms of those patients. A reduction in the P-gp function has also been described in Parkinson's patients (9,58), in patients with Creutzfeldt-Jakob's disease (64), and multiple sclerosis patients (65). Although the molecular mechanism causing this decrease in the P-gp function remains poorly understood (7,58), P-gp inducers may be beneficial in the treatment of these diseases.

Recently, a new class of P-gp modulators called P-gp activators has been discovered. These compounds temporarily activate the P-gp function without increase the P-gp expression. P-gp activators bind to the transporter and cause a conformational change that promotes the transport of a P-gp substrate bound to another binding site (6,47). In vitro studies have already shown the ability of several thioxanthone derivatives to modulate the P-gp transporter by enhancing its function. These compounds can be used as potential treatments to reverse intoxications caused by a P-gp substrate (47,66).

[¹⁸F]MC225 PET scans can be used to identify potential P-gp modulators which can be used for the treatment of diseases where the P-gp function is altered. Inhibitors may be used for overcoming drug resistance in brain tumors, intractable epilepsy, or in non-responsive patients

with psychiatric disorders (48,51), and the proposed approaches to cause a local inhibition of the P-gp function can also be used to improve the brain delivery of drugs to specific regions (52). Inducers can be promising therapeutics for the treatment of diseases where the P-gp function is decreased such as Alzheimer's, Parkinson's, multiple sclerosis, and activators can help in intoxications caused by P-gp substrates such as paraquat (herbicide) (6,47,67). [^{18}F]MC225 PET may allow the monitoring of the efficacy of these novel treatments *in vivo* in disease animal models.

P-gp function versus P-gp expression

It is important to distinguish whether a compound alters the P-gp function or the P-gp expression to fully understand the mechanism of action of that compound and its potential use. P-gp inducers increase the P-gp expression, but this action does not always imply an increase in the P-gp function, and the opposite occurs with the P-gp activators. Thus, it is important to simultaneously evaluate both processes *in vivo*. The [^{18}F]MC225 scans only allow the quantification of the P-gp function since the radiotracer does not bind to the transporter as it acts as a P-gp substrate. It is important to develop new P-gp tracers able to measure the expression of the transporter. The first approach was to radiolabel P-gp inhibitors which may bind to the P-gp transporter, however, this strategy failed because those P-gp inhibitors acted as P-gp substrates at tracer amounts (68,69). Recently, Verbeek and colleges have published the synthesis and *in vivo* evaluation in mice and rats of a new compound called [^{18}F]4FIMPTC that may be a promising radiotracer that binds to the P-gp transporter and thus allows the evaluation of P-gp expression levels *in vivo* (70). Further research is needed to verify the properties of this novel P-gp tracer.

Use of [^{18}F]MC225 in drug development and preclinical research

The adequate reproducibility of the [^{18}F]MC225 PET scans in rats, the ability of this tracer to measure both increases and decreases in the P-gp function and the radiolabeling of the tracer with fluorine-18 which allow the distribution of the tracer to other PET centers, make [^{18}F]MC225 an ideal tracer to use in (pre)clinical research concerning the assessment of the P-gp function.

In most cases, drugs developed for CNS diseases, do not reach the market due to unexpected toxicities or their implication in DDIs (71). Since many compounds can act as P-gp inducers or inhibitors, the concomitant administration of these P-gp modulators together with a pharmaceutical that is a P-gp substrate can lead to changes in the bioavailability of the drug to its target organ (72,73). To avoid undesired side effects, the pharmaceutical industry can identify potential interactions *in vivo* with the P-gp transporter at the early stage of the drug development

process by using [^{18}F]MC225 PET scans. For example, P-gp function can be studied at baseline conditions and after the administration of the drug under evaluation in the same animal to assess its effect on the P-gp function *in vivo*.

On the other hand, [^{18}F]MC225 PET scans can be used to elucidate the role of the P-gp transporter in several brain disorders. Animal models of Alzheimer's and Parkinson's disease can be used to study the implication of this transporter in the onset or development of these diseases. The P-gp function can also be assessed in animals with brain tumors or with an induced status epilepticus to clarify the contribution of this transporter to drug resistance.

Clinical [^{18}F]MC225 PET scans

[^{18}F]MC225 PET scans may allow the identification of patients with an increased or decreased P-gp function. For instance, the translation of [^{18}F]MC225 to humans may allow the quantification of the P-gp function in patients with Alzheimer's. To date, it is not known if the decrease in the P-gp function is the cause or the consequence of the development of Alzheimer's disease. The performance of [^{18}F]MC225 PET scans in patients with mild cognitive impairment, patients at the early stage of the disease (74), may provide insight into the underlying biological process. On the other hand, [^{18}F]MC225 PET scans may allow the quantification of increases in the P-gp function in non-responsive patients as occur in intractable epilepsy or treatment-resistant depression.

However, before the use of [^{18}F]MC225 in patients, its kinetics must be evaluated in healthy volunteers to assess the normal distribution of the tracer and the reproducibility of these studies should also be evaluated. Next, the kinetics of [^{18}F]MC225 must be studied after the administration of a P-gp inhibitor, to know if the tracer can detect changes in the P-gp function at the BBB of humans. If [^{18}F]MC225 shows similar kinetics to those described in previous animal studies, the P-gp function of those patients with neurodegenerative diseases or drug resistance issues can be monitored to find out the implication of this transporter in those pathologies and select the most appropriate medication.

Besides that, the introduction of the total-body PET-camera would allow the assessment of radiotracer distribution at different organs and tissues of the human body simultaneously. Conventional PET scanners can only scan a certain part of the human body that enters inside the field of view of the camera, meanwhile, total-body PET-camera are scanners that can image the whole body of a subject at once. Therefore, total-body PET-cameras would allow studying inter-connections in systemic diseases (75). Recently, a decreased intestinal P-gp expression has been found in Alzheimer's disease patients compared to elder subjects without dementia, which highlights the importance of the microbiota-gut-brain axis (76). Dynamic whole-body PET scans

with [^{18}F]MC225 would allow studying intestinal and cerebral P-gp function simultaneously and may provide new insights about the gut-brain axis. Additionally, other diseases have been related to changes in the microbiota as autism, depression, anxiety, and Parkinson's disease (77) and it would be of interest to study the role of the P-gp transporter in these diseases at different organs.

Development of new PET tracers targeting different components at BBB

Besides the P-gp transporter, other ATP binding Cassette (ABC) transporters have been involved in the onset and development of different brain disorders and drug-resistances issues. Impairments in the Breast Cancer Resistance Protein (BCRP) and Multidrug Resistance Protein-1 (MRP1) function and expression have been found in Alzheimer's, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's, and epilepsy patients (78). For this reason, it is also of interest to develop novel PET tracers targeting different ABC transporters. However, the development of BCRP and MRP1 PET tracers has faced some challenges mainly due to the overlap in binding-substrates. Most of the BCRP and MRP1 substrates are also substrates of the P-gp transporter, complicating the development of specific BCRP and MRP1 PET tracers (68,79).

In addition to the ABC transporters, several transporters located at the BBB have been associated with the onset or development of several diseases such as stroke, epilepsy, multiple sclerosis, and neurodegenerative diseases (58,80). In the case of Alzheimer's diseases, a decrease in the levels of glucose endothelial transporter (GLUT-1) has been found. Also, a decrease was found in the expression levels of the low-density lipoprotein receptor-related protein 1 (LRP-1) which is a receptor involved in the transport of A β out of the brain. In contrast, the expression of the receptor for advanced glycation end-products (RAGE) was up-regulated in these patients (81–83) (Figure 2). All these alterations support the BBB structure as an important player and thus a potential candidate for novel therapies. For this reason, it is of interest to develop PET tracers targeting different transporters or receptors at the BBB to study the progression of a disease and discover new therapeutic targets. In the case of RAGE, some groups have already developed some tracers to study the expression of these receptors. However, preliminary evaluation of these PET candidates was not successful and further studies are needed to develop more specific tracers (84,85).

Figure 2: Potential targets for the diagnosis or treatment of Alzheimer's disease located at the BBB.

Concluding remarks

The content of this thesis supports the translation of [^{18}F]MC225 to the clinical settings since the tracers have been able to measure the P-gp function at the BBB and it has shown adequate pharmacokinetic characteristics. Moreover, this tracer is highlighted as an improved P-gp tracer due to its ability to measure both decreases and increases in the P-gp function. The radiolabeling of the tracer with fluorine-18 also represents an advantage. The longer half-life of this isotope ($T_{1/2}=110$ min) compared to carbon-11 ($T_{1/2}=20$ min) would allow the distribution of the tracer to other PET tracers without an on-site cyclotron. The good reproducibility of the [^{18}F]MC225 PET scans may allow the monitoring of the P-gp function at different stages of the disease as well as before and after the administration of treatments. Further studies in humans are needed to verify the adequate pharmacokinetics of the tracer and its clinical value.

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Chapter 10

Summary

Summary

In previous studies, the Positron Emission Tomography (PET) tracer [^{18}F]MC225 has shown an adequate specificity and sensitivity towards the P-glycoprotein (P-gp) transporter in mice and rats (1,2). Furthermore, those studies showed a higher brain [^{18}F]MC225 uptake at baseline when compared with (*R*)-[^{11}C]Verapamil, considered as the gold standard radiotracer for imaging the P-gp function (1,2). Although (*R*)-[^{11}C]Verapamil can measure decreases in the P-gp function, its low uptake at baseline hampers its use to measure increases in the P-gp function (3), since an increase in the P-gp function will further decrease the radiotracer uptake inside the brain. In this regard, the highest [^{18}F]MC225 uptake at baseline may allow the evaluation of increases in the P-gp function, suggesting that [^{18}F]MC225 may be a more suitable tracer for measuring both decreases and increases of the P-gp function. This PhD thesis aims to expand the current knowledge on [^{18}F]MC225, collect sufficient information to verify the described properties, and thus, supporting the clinical translation of the tracer.

First, **Chapter 2** discusses the role of the P-gp transporter and other ATP Binding Cassette (ABC) transporters in the onset and progression of several neurodegenerative diseases and their implication in drug resistance issues. Moreover, this chapter also summarizes the current available P-gp PET tracers and their potential clinical use.

The reproducibility of the [^{18}F]MC225 PET scans was evaluated in **Chapter 3**. Longitudinal PET studies were performed for evaluating P-gp function at different time points, for instance, before and after the onset of a disease or treatment administration. In addition, since preclinical PET imaging involves the use of anesthesia, the impact of repeated exposures to isoflurane-anesthesia on the brain uptake of [^{18}F]MC225 was also assessed. Thus, a group of animals was subjected to test-retest PET scans with [^{18}F]MC225, and another group of animals underwent PET scans after being previously exposed to anesthesia. This study did not find significant changes in the influx constant (K_1), which is considered the best parameter to measure the P-gp function in rats (2). These results were supported by Western Blot experiments which did not find any significant increase in the P-gp expression of animals pre-exposed to anesthesia. The study concludes that the isoflurane-anesthesia may affect the distribution of the radiotracer altering the k_2 and V_T values, however, isoflurane-anesthesia does not alter the P-gp function and expression at the BBB. Therefore, longitudinal studies with [^{18}F]MC225 may be possible, but only if K_1 is used to estimate the P-gp function.

Although the pharmacokinetics of [^{18}F]MC225 were already studied in mice and rats (1,2), it is suggested to validate the efficacy of new compounds in at least two different species as part of the drug development process. Moreover, it is crucial to choose species that possess high similarity to humans in order to support an adequate translation from preclinical studies to clinical

(4). For this, a pharmacokinetic evaluation of [^{18}F]MC225 was performed in non-human primates, due to its similarity to humans in terms of P-gp expression level (5), before and after the inhibition of the P-gp function (**Chapter 4**). [^{18}F]MC225 PET data were analyzed using different tissue compartmental models (TCM) and various scan durations (10- to 91-min scan duration). The effect of the scan duration and P-gp inhibition on the kinetic parameters was assessed. The 1-TCM showed to be the best-fitting model for short scan duration, while the 2-TCM should be used for long scans. In all tested scan durations, K_1 and V_T values of all the brain regions significantly increased after the P-gp inhibition. K_1 was the parameter most affected by the P-gp inhibition and was selected as the best parameter for measuring the P-gp function also due to the stability of its estimations regardless of the scan duration. Therefore, the 1-TCM K_1 constant was our parameter of choice for measuring the P-gp function at the BBB of non-human primates. The V_T and K_1 calculated using the 2-TCM were also suitable options when using long scan durations. These conclusions are in line with previous studies performed in rats and mice and open the door to perform further human studies.

Despite the kinetics of (*R*)-[^{11}C]Verapamil have been extensively studied in small rodents and humans (6–9), a pharmacokinetic evaluation of (*R*)-[^{11}C]Verapamil in non-human primates has not been done previously. The aim of **Chapter 5** was to establish (*R*)-[^{11}C]verapamil as a reference P-gp radiotracer for comparison of newly developed P-gp PET radiotracers in non-human primates. To this aim, the kinetics of (*R*)-[^{11}C]verapamil were evaluated in the same non-human primates before and after the inhibition of the P-gp function. Furthermore, we evaluated the effect of the scan durations (10- to 91-min) and the P-gp inhibition on the kinetic parameters. Our results suggested that the 1-TCM should be the model of choice to analyze PET data of (*R*)-[^{11}C]verapamil in non-human primates at baseline and after-inhibition scans regardless of the scan duration. The analysis found that estimations of K_1 values remained relatively constant, while V_T and k_2 estimations were affected by the scan duration. After the P-gp inhibition, K_1 and V_T values increased for all the brain regions and scan durations, whereas k_2 values decreased for all scan durations except for the 91-min. V_T was the most affected parameter after inhibition. In line with previous studies performed in different animal species, the tracer was quickly metabolized. Thus, our results suggest that short scans should be used to avoid the interference of radio-metabolites on the kinetic quantification. In summary, our study supports the use of K_1 , calculated with 1-TCM, and using short PET scans (10- to 30-min) as a suitable parameter to measure the P-gp function at the BBB of non-human primates.

In **Chapter 6** we use the data obtained in **Chapters 4** and **5** to perform a direct comparison between [^{18}F]MC225 and (*R*)-[^{11}C]Verapamil. To this aim, the 30-min PET data of both radiotracers were fitted to a 1-TCM following the recommendations from previous publications (10,11). Kinetic parameters as K_1 , V_T , and k_2 were compared between the radiotracers at baseline

and after inhibition. Also, regional differences in radiotracer uptake were analyzed, and simplified methods to quantify the P-gp function were proposed. At baseline scans, the V_T values of [^{18}F]MC225 were higher and k_2 values were lower than those of (*R*)-[^{11}C]Verapamil. K_1 values were not significantly different between tracers. After the inhibition of the P-gp function, the K_1 and k_2 values of (*R*)-[^{11}C]Verapamil were higher than those of [^{18}F]MC225, while the V_T values of both tracers did not show significant differences. The inhibition of the P-gp function increased the K_1 and V_T values for both tracers, and in the case of (*R*)-[^{11}C]Verapamil, the inhibition also caused a decrease in k_2 values. This comparison study suggests that the V_T values of both tracers are equally sensible to measure decreases in the P-gp function at the BBB of non-human primates. Regarding (*R*)-[^{11}C]Verapamil, the study suggests that the V_T may be a more adequate parameter to measure the P-gp function than the K_1 . The study also demonstrated significant regional differences between tracers at baseline, which disappeared after inhibition. The results from both tracers indicate that subcortical regions may present a higher P-gp function than the frontal cortex and cerebellum. As a simplified method, we found that the positive slope of the SUV-TAC was positively correlated to the K_1 and V_T for both tracers. Moreover, the results confirm the higher baseline V_T of [^{18}F]MC225, which, as mentioned earlier, may allow the quantification of increases in the P-gp function, although further studies are still needed to verify this ability.

The current synthesis of [^{18}F]MC225 involves two steps. The aim of **Chapter 7** was to optimize the production of [^{18}F]MC225, currently requiring the distillation of [^{18}F]fluoroethylbromide as an intermediate step. To this aim, a new precursor that allows the production of [^{18}F]MC225 via direct radiofluorination was developed. A mesylate precursor was successfully produced, however, this compound was not stable due to the addition of the leaving group (mesylate). Eventually, a salt of the mesylate precursor was produced which improved the stability of the compound for a longer time. However, the final mesylate precursor presented some impurities, and although several methods were applied to further purify the compound, these efforts were unsuccessful. Nevertheless, [^{18}F]MC225 was successfully produced by the one-step synthesis using the developed precursor, and the radiosynthesis was automated in a radiosynthesis module (IBA Synthera).

Finally, in **Chapter 8** [^{18}F]MC225 was used to evaluate the effect of a new P-gp inducer (MC111) on the P-gp transporter function. A decline in the P-gp function at the BBB can increase the concentration of neurotoxic compounds inside the brain, which can be related to the onset and progression of several neurodegenerative diseases, and, P-gp inducers are currently considered potential candidates for the treatment of such disorders (12,13). MC111 has been identified as a P-gp and BCRP inducer by *in vitro* studies and its dual activity may be beneficial for the treatment of such neurological conditions. Therefore, our study aimed at verifying the P-gp induction effect of MC111 *in vivo* using PET imaging. Therefore, healthy rats were injected with either a vehicle

solution (control group), 4.5 mg/kg of MC111 (low-dose group), or 6 mg/kg of MC111 (high-dose group) one day before the [^{18}F]MC225 PET scan. After the PET scan, animals were sacrificed and brain tissue was collected to assess the P-gp expression using western blot. Our results showed that the administration of 6 mg/kg of MC111 significantly decreased the K_1 and V_T values of [^{18}F]MC225 in treated rats compared to control rats which indicates an increase in the P-gp function due to the administration of MC111. However, the western blot results did not find any increase in the P-gp expression levels in treated rats. Several mechanisms can induce the P-gp expression levels at the surface of the cells. Since the P-gp expression levels of the whole brain did not increase after the administration of MC111, it is suggested that MC111 may increase the P-gp expression level in the surface of the endothelial brain cells by relocating intracellular P-gp protein to the plasma membrane (14,15). Overall, the decrease in K_1 and V_T values of [^{18}F]MC225 indicates an increase in the P-gp function at the BBB of treated rats. So, these results also verify the ability of [^{18}F]MC225 to measure increases in the P-gp function of rats.

To conclude, the results presented in this thesis provided sufficient information to support the clinical translation of [^{18}F]MC225. [^{18}F]MC225 has shown improved pharmacokinetic characteristics compared to the gold standard P-gp PET tracer (*R*)-[^{11}C]verapamil. The main advantage of [^{18}F]MC225 is the highest baseline V_T values and its ability to measure increases in the P-gp function. [^{18}F]MC225 has the potential to become the first radiofluorinated tracer able to measure both decreases and increases in the P-gp function at the BBB.

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Chapter 11

Nederlandse samenvatting

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In eerdere preklinische studies heeft Positron Emission Tomography (PET) tracer [^{18}F]MC225 goede sensitiviteit en specificiteit laten zien voor de P-glycoproteïne (P-gp) transporter in muizen en ratten (1,2). In deze studies werd bij een normale P-gp functie een hogere [^{18}F]MC225 uptake in het brein gevonden in vergelijking met de opname van (*R*)-[^{11}C]Verapamil, de gouden standaard radiotracer voor het meten van de P-glycoproteïne functie (1,2). Alhoewel het mogelijk is om met (*R*)-[^{11}C]Verapamil een afname in P-gp functie te meten, belemmert de hoge uptake bij een normale P-gp functie het gebruik van [^{11}C]verapamil voor het meten van een toename in P-gp functie (3), aangezien een toename in P-gp functie zal leiden tot een verdere afname van radiotracer uptake in het brein. In deze zin zou de hogere opname van [^{18}F]MC225 in het brein bij een normale P-gp functie het mogelijk maken ook toename in P-gp functie te evalueren. Dit maakt [^{18}F]MC225 een meer geschikte PET radiotracer dan [^{11}C]Verapamil voor het meten van zowel toe- als afname van het P-gp functie. Dit proefschrift heeft als doel de huidige kennis omtrent [^{18}F]MC225 uit te breiden en voldoende informatie te verzamelen om de eerder beschreven eigenschappen te verifiëren, ter ondersteuning van de klinische translatie van deze tracer.

Eerst zal in **Hoofdstuk 2** de rol van de P-gp transporter en andere ATP Binding Cassette (ABC) transporters bij de ontwikkeling en progressie van enkele neurodegeneratieve aandoeningen behandeld worden. Ook wordt in dit hoofdstuk de functie van de P-gp transporter en ABC transporters in het algemeen bij de ontwikkeling van medicijn-resistentie belicht. Tevens geeft dit hoofdstuk een samenvatting van de huidige beschikbare P-gp PET tracers voor het meten van de P-gp functie en hun klinische toepasbaarheid.

De reproduceerbaarheid van [^{18}F]MC225 PET scans werd geëvalueerd in **Hoofdstuk 3**. Longitudinale PET studies werden uitgevoerd om de P-gp functie op verschillende tijdstippen te meten, onder andere voor en na de ontwikkeling van ziekte en voor en na het toedienen van een behandeling. Omdat bij preklinische PET studies over het algemeen gebruik wordt gemaakt van anesthesie, werd ook de impact van herhaaldelijke blootstelling aan isofluraan op de uptake van [^{18}F]MC225 in de hersenen onderzocht. In dit onderzoek werd één groep dieren opgenomen in een test-retest setting met [^{18}F]MC225 PET scans en één groep onderging de PET scans na blootstelling aan anesthesie. In deze studie werden geen significante verschillen gevonden in de influx constante (K_1), de meest optimale parameter om P-gp functie in ratten te meten (2). De resultaten van dit onderzoek werden ondersteund door Western Blot experimenten, waarin geen significante toename van P-gp expressie werd gevonden bij de dieren die waren blootgesteld aan anesthesie. De conclusie van deze studie is dat het gebruik van isofluraan-anesthesie mogelijk effect heeft op de distributie van de radiotracer, waarbij veranderingen in de k_2 en V_T optreden,

maar isofluraan-anesthesie geen effect heeft op de functie en expressie van P-gp op de bloed-hersenbarriere. Hieruit werd geconcludeerd dat longitudinale studies met [^{18}F]MC225 mogelijk zijn, wanneer K_1 gebruikt wordt om de P-gp functie te beschrijven.

Alhoewel de farmacokinetiek van [^{18}F]MC225 eerder onderzocht is in muizen en ratten (1,2), wordt gesuggereerd dat de werkzaamheid van nieuwe middelen in ten minste twee verschillende diersoorten geëvalueerd moet worden als onderdeel van het ontwikkelingsproces. Voor een adequate translatie van preklinische naar klinische studies is het cruciaal diersoorten te onderzoeken die dicht bij de mens staan (4). Dit in overweging nemende, werd een farmacokinetische evaluatie van [^{18}F]MC225 opgezet bij non-human primates voor en na inhibitie van de P-gp functie (**Hoofdstuk 4**). Er werd gekozen voor een studie in non-human primates vanwege hun gelijkenis met de mens op gebied van P-gp functie en expressie (5). [^{18}F]MC225 data werd geanalyseerd met gebruik van verschillende Tissue Compartmental Models (TCM) en verschillende scanduraties (10 tot 91 minuten scantijd). Het effect van zowel de verschillende scantijden als van de inhibitie van de P-gp functie werden bestudeerd. Het 1-TCM bleek de beste fit te geven voor korte scantijden, terwijl het 2-TCM de beste uitkomsten gaf voor langere scantijden. Bij alle geëvalueerde scantijden namen zowel de K_1 als de V_T waarden significant toe na inhibitie van de P-gp functie. K_1 was de parameter die de meeste verandering liet zien na inhibitie van de P-gp functie en werd geselecteerd als beste parameter voor het meten van de P-gp functie, ook vanwege de stabiliteit van de K_1 metingen, ongeacht de scanduur. Deze overwegingen in ogenschouw nemend, werd de 1TCM K_1 constante de parameter van keuze voor het meten van de P-gp functie op de bloed-hersenbarriere van non-human primates. De V_T en K_1 vastgesteld met het 2-TCM werden ook gezien als geschikte parameters, echter alleen voor langere scantijden. De conclusies van deze studie komen overeen met eerdere studies uitgevoerd in ratten en muizen en laten de potentie van deze radiotracer zien voor verder evaluatie van [^{18}F]MC225 in de mens.

Ondanks uitgebreide eerdere studies naar de farmacokinetiek van (*R*)-[^{11}C]Verapamil in preklinische en klinische studies (6-9), is de farmacokinetiek van (*R*)-[^{11}C]Verapamil in non-human primates niet eerder bestudeerd. Het doel van **Hoofdstuk 5** was de evaluatie van (*R*)-[^{11}C]Verapamil als referentie P-gp radiotracer om nieuw ontwikkelde P-gp PET tracers in non-human primates mee te vergelijken. De kinetiek van (*R*)-[^{11}C]Verapamil werd geëvalueerd in non-human primates voor en na inhibitie van de P-gp functie. Ook werd de invloed van de scanduur (10 tot 91 minuten) en het effect van de P-gp inhibitie op de kinetische parameters geëvalueerd. Deze studie laat zien dat het 1-TCM het model van eerste keuze is om de P-gp functie te meten met (*R*)-[^{11}C]Verapamil PET, zowel voor de scans voor als na P-gp inhibitie, onafhankelijk van de scanduur. Uit de resultaten bleek dat de K_1 waarden relatief constant bleven, terwijl de V_T en k_2 waarden veranderingen lieten zien bij gebruik van een kortere of langere scanduur. Na inhibitie

van P-gp namen de K_1 en V_T waarden voor alle breinregio's en scantijden toe, terwijl de k_2 waarden voor alle scantijden afnamen met uitzondering van de 91 minuten scan. De V_T liet de grootste veranderingen zien na inhibitie van de P-gp functie. Ook werd een snelle metabolisatie van (R) - $[^{11}\text{C}]$ Verapamil gevonden, overeenkomstig eerdere literatuur. De resultaten van de huidige studie, suggereren dat korte scantijden gebruikt moeten worden, om interferentie van radio-metabolieten op de kwantificatie uitkomsten van deze tracer te vermijden. Concluderend: de studie ondersteunt het gebruik van K_1 , vastgesteld met het 1-TCM, bij korte scantijden (10-30 minuten) voor het meten van P-gp functie op de bloed-hersenbarrière van non-human primates.

In **Hoofdstuk 6** werd de data uit **hoofdstuk 4 en 5** gebruikt om een directe vergelijking te maken tussen $[^{18}\text{F}]\text{MC225}$ en (R) - $[^{11}\text{C}]\text{Verapamil}$. Hiervoor werd PET data van beide radiotracers geëvalueerd met behulp van een 1-TCM (gebaseerd op 30 minuten scantijd), zoals aanbevolen in eerdere literatuur (10, 11). Kinetische parameters als K_1 , V_T en k_2 van zowel baseline scans als scans na inhibitie van de P-gp functie werden vergeleken. Ook werden regionale verschillen in tracer uptake geanalyseerd en gesimplificeerde methoden om de P-gp functie te kwantificeren bestudeerd. De studie liet zien dat in vergelijking met $[^{11}\text{C}]\text{Verapamil}$ de $[^{18}\text{F}]\text{MC225}$ baseline scans hogere V_T waarden en lagere k_2 waarden gaven. Er bestonden geen significante verschillen tussen de K_1 waarden van $[^{18}\text{F}]\text{MC225}$ en $[^{11}\text{C}]\text{Verapamil}$. Na inhibitie van de P-gp functie, waren de zowel de K_1 als k_2 waarden van (R) - $[^{11}\text{C}]\text{Verapamil}$ hoger dan die van $[^{18}\text{F}]\text{MC225}$, terwijl de V_T parameter geen significante verschillen liet zien tussen beide tracers. Inhibitie van de P-gp functie deed de K_1 en V_T waarden voor beide tracers toenemen en bij toediening van (R) - $[^{11}\text{C}]\text{Verapamil}$ werd hiernaast ook een afname in k_2 waarden gevonden. Deze vergelijkingsstudie suggereert dat de V_T waarden van beide tracers in gelijke mate gevoelig zijn voor het meten van afnames in P-gp functie op de bloed-hersenbarrière van non-human primates. Voor (R) - $[^{11}\text{C}]\text{Verapamil}$ laat de huidige studie zien dat de V_T een meer adequate parameter is voor het meten van de P-gp functie dan K_1 . De studie laat tevens zien dat er significante regionale verschillen bestaan tussen de twee tracers voor de baseline scans, maar dat deze verschillen verdwijnen na inhibitie van de P-gp functie. Beide tracers laten een hogere P-gp functie zien in de subcorticale regio's, in vergelijking met de frontale cortex en het cerebellum. Bij de evaluatie van vereenvoudigde analyse methoden voor het meten van de P-gp functie, werd gevonden dat een positieve helling van de SUV-TAC positief gecorreleerd was met de K_1 en V_T van beide tracers. Ook bevestigen de resultaten van deze studie een hogere baseline uptake van $[^{18}\text{F}]\text{MC225}$ vergeleken met (R) - $[^{11}\text{C}]\text{Verapamil}$. Dit betekent dat $[^{18}\text{F}]\text{MC225}$ naast het meten van afname in P-gp functie in de toekomst wellicht ook toename van P-gp functie zou kunnen meten. Verdere studies zijn nodig om deze hypothese te bevestigen.

De huidige synthese van $[^{18}\text{F}]\text{MC225}$ wordt in twee stappen uitgevoerd. Het doel van **Hoofdstuk 7** was om de synthese van $[^{18}\text{F}]\text{MC225}$, waarin een tussenstap wordt gemaakt vanwege de vereiste

distillatie van [^{18}F]fluoroethylbromide, te optimaliseren. Om dit te bereiken werd een nieuwe precursor ontwikkeld, die de synthese van [^{18}F]MC225 via directe radio-fluorinatie mogelijk maakte. Een mesylaat precursor werd succesvol geproduceerd, maar bleek niet stabiel genoeg door de toevoeging van de ‘leaving group’ (mesylaat). Uiteindelijk werd een zout van de mesylaat precursor geproduceerd, waardoor de stabiliteit van de verbinding langer kon worden gewaarborgd. Deze mesylaat precursor liet echter enige onzuiverheid zien, en meerdere methodes om de verbinding te zuiveren bleken niet succesvol. Niettemin werd [^{18}F]MC225 succesvol geproduceerd volgens de één-staps-synthese met gebruik van de ontwikkelde precursor en de radiosynthese werd geautomatiseerd in de radiosynthese-module (IBA Synthara).

Tenslotte werd in **Hoofdstuk 8** [^{18}F]MC225 gebruikt om het effect van een nieuwe P-gp inducer (MC111) op de P-gp transporter-functie te evalueren. Een afname van P-gp functie op de bloed-hersenbarriere kan leiden tot een verhoogde concentratie neurotoxische stoffen in het brein. Deze verhoogde concentraties neurotoxische stoffen zijn gerelateerd aan de ontwikkeling en progressie van verschillende neurodegeneratieve aandoeningen en de toediening van P-gp inducers wordt momenteel dan ook overwogen als potentiële behandelingsmogelijkheid voor deze aandoeningen (12, 13). *In vitro* studies hebben aangetoond dat MC111 zowel een P-gp als BCRP inducer is en dat deze duale activiteit kan bijdragen bij de behandeling van deze neurologische aandoeningen. De huidige studie heeft als doel het P-gp inductie effect van MC111 *in vivo* te evalueren met gebruik van PET imaging. Gezonde ratten kregen een injectie met ofwel een vehicle oplossing (controle groep) of werden één dag voor de PET scan behandeld met 4.5 mg/kg MC111 (lage dosering) of 6 mg/kg MC111 (hoge dosering). Na de scan werden de dieren opgeofferd waarna hersenweefsel werd verzameld om de P-gp expressie te evalueren door middel van western blot. De resultaten van deze studie laten zien dat de toediening van 6 mg/kg MC111 leidt tot een significante afname van K_1 en V_T waarden van [^{18}F]MC225 in ratten in vergelijking met de controle groep. Dit duidt op een toename van P-gp functie door toediening van MC111. Echter, de resultaten van de western blot lieten geen significante toename van P-gp expressie zien in de ratten behandeld met MC111. Verschillende mechanismen kunnen P-gp expressie op het celoppervlak induceren. Aangezien er geen toename van P-gp expressie levels gevonden werd na toediening van MC111, wordt gesuggereerd dat MC111 wellicht slechts het P-gp expressie level aan het oppervlak van de endotheelcellen in de hersenen doet toenemen door relocatie van intracellulair P-gp eiwit naar het plasma membraan. (14, 15). Over het algemeen, duidt de afname van K_1 en V_T op een toename van P-gp functie op de bloed-hersenbarriere van de met MC111 behandelde ratten. Deze resultaten verifiëren tevens de mogelijkheid om met [^{18}F]MC225 een toename van P-gp functie te meten in ratten.

Samenvattend, geven de resultaten gepresenteerd in dit proefschrift voldoende informatie om de klinische translatie van [^{18}F]MC225 te ondersteunen. [^{18}F]MC225 laat verbeterde kinetische

eigenschappen zien in vergelijking met de gouden standaard [^{11}C]Verapamil. Het grootste voordeel van [^{18}F]MC225 wordt gevonden in de hogere V_T waarden in baseline scans en het vermogen om toename in P-gp functie te meten. [^{18}F]MC225 heeft de potentie om de eerste fluorine-gelabelde radiotracer te worden voor het meten van zowel toe- als afname van de P-gp functie op de bloed-hersenbarriere.

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Chapter 12

Resumen

Resumen

En estudio previos, el radiotrazador para Tomografía de Emisión de Positrones (PET, por sus siglas en inglés), [^{18}F]MC225, ha mostrado una adecuada especificidad y sensibilidad por el transportador de glicoproteína-P (P-gp por su abreviación en inglés) en ratones y ratas (1,2). Además, estos estudios mostraron una mayor captación de [^{18}F]MC225 en el cerebro en condiciones basales en comparación con (R)-[^{11}C]Verapamil, radiotrazador que es considerado de referencia para la evaluación de la función de los transportadores de P-gp (1,2). Aunque (R)-[^{11}C]Verapamil es capaz de medir disminuciones de la función de los P-gp, su baja penetración cerebral en condiciones normales impide que este radiotrazador pueda medir aumentos de la función de los P-gp (3), ya que un aumento en la función hará que la penetración del radiotrazador disminuya aún más dentro del cerebro. En este aspecto, la mayor penetración de [^{18}F]MC225 en condiciones basales puede que permita la evaluación de aumentos en la función de los P-gp, lo que sugiere que [^{18}F]MC225 puede ser un radiotrazador más apropiado para medir tanto disminuciones como aumentos de la función de los P-gp. Esta tesis doctoral tiene como objetivo ampliar el actual conocimiento sobre el radiotrazador [^{18}F]MC225, recopilar suficiente información para verificar las propiedades previamente descritas y así poder apoyar la translación del radiofármaco a la clínica.

En primer lugar, el **Capítulo 2** discute el papel de los transportadores de P-gp y otros transportadores dependientes de ATP (ABC transportadores por sus siglas en inglés) en la aparición y progresión de diferentes enfermedades neurodegenerativas y su implicación en procesos de fármaco-resistencia. Además, este capítulo también menciona los radiotrazadores PET actualmente disponibles para medir la función de los P-gp y su potencial aplicación en la clínica.

La reproducibilidad de los escáneres PET con [^{18}F]MC225 fue evaluada en el **Capítulo 3**. Se realizaron estudios PET longitudinales para la evaluación de la función de los P-gp a diferentes tiempos. Además, dado que las imágenes de PET preclínicas implican el uso de anestesia, también se evaluó el impacto de repetidas exposiciones al anestésico isoflurano en la captación cerebral del radiotrazador [^{18}F]MC225. Para ello, a un grupo de animales se les realizó estudios PET test-retest con [^{18}F]MC225, y a otro grupo de animales se les realizó un escáner PET después de haber estado previamente expuesto a anestesia. Este estudio no encontró cambios significativo en los valores de la constante de flujo de entrada (K_1), la cual se considera el mejor parámetro para medir la función de los P-gp en ratas (2). Estos resultados fueron respaldados por otros experimentos en los que se utilizó la técnica de Western Blot, los cuales tampoco encontraron ningún incremento significativo en la expresión de los P-gp en animales previamente expuestos a anestesia. El estudio concluye que el anestésico isoflurano puede afectar la distribución del radiotrazador alterando los

valores de k_2 y V_T , sin embargo, el isoflurano no altera la función ni la expresión de los P-gp en la barrera hematoencefálica (BHE). Por ello, es posible la realización de estudios longitudinales con [^{18}F]MC225 siempre y cuando se utilice la K_1 para estimar la función de los P-gp.

Aunque la farmacocinética de [^{18}F]MC225 ha sido previamente estudiada en ratones y ratas (1,2), en el proceso de desarrollo de medicamentos, es conveniente validar la eficacia de nuevos compuestos en al menos dos especies diferentes, además, es esencial elegir especies que posean un alto grado de similitud con los humanos para poder respaldar adecuadamente la translación desde etapas preclínicas a clínicas (4). Por esta razón, se realizó una evaluación farmacocinética de [^{18}F]MC225 en primates, debido a su similitud con los humanos en cuanto al nivel de expresión de los P-gp (5), antes y después de la inhibición de la función de los P-gp (**Capítulo 4**). Los datos de los escáneres PET con [^{18}F]MC225 fueron analizados utilizando diferentes modelos de tejidos compartimentales (TCM por sus siglas en inglés) y varias duraciones de exploración (duración de exploración de 10- a 91- min). Se examinó el efecto de la exploración y la inhibición de los P-gp sobre los parámetros farmacocinéticos. El mejor ajuste de los datos fue obtenido con el 1-TCM para escaneos de poca duración, mientras el 2-TCM debe usarse para escaneos largos. En todas las duraciones de exploración examinadas, los valores de K_1 y V_T de todas las regiones cerebrales aumentaron significativamente después de la inhibición de los P-gp. La K_1 fue el parámetro más afectado por la inhibición y fue seleccionado como el mejor parámetro para medir la función de los P-gp debido a la estabilidad de sus estimaciones independientemente de la duración del escaneo. Por lo tanto, proponemos la utilización de la K_1 calculada utilizando el 1-TCM como mejor parámetro para medir la función de los P-gp en la BHE de primates. El V_T y la K_1 calculados utilizando el 2-TCM también fueron opciones adecuadas cuando se utilizaron escaneos de larga duración. Las conclusiones de este estudio concuerdan con los resultados obtenidos en ratas y ratones e incitan la realización de estudios en humanos.

A pesar de que la farmacocinética de (R)-[^{11}C]Verapamil se ha estudiado extensamente en roedores y humanos (6–9), la cinética de (R)-[^{11}C]Verapamil no se ha evaluado aun en primates. El objetivo del **Capítulo 5** es establecer (R)-[^{11}C]Verapamil como un radiotrazador de referencia para medir la función de los P-gp y así poder compararlo con la cinética de nuevos radiotrazadores PET en primates. Con este fin, la farmacocinética de (R)-[^{11}C]Verapamil se evaluó en primates antes y después de la inhibición de la función de los P-gp. Además, también se evaluó el efecto del uso de diferentes duraciones de exploración (de 10- a 91-min) y la inhibición de la función de los P-gp sobre los parámetros farmacocinéticos. Nuestros resultados sugieren que el 1-TCM debe ser el modelo de elección para analizar los datos PET de (R)-[^{11}C]Verapamil en primates tanto en condiciones basales como después de la inhibición de los P-gp e independientemente de la duración de la exploración. El análisis mostró que la estimación de los valores de K_1 permanecieron relativamente estables, mientras que las estimaciones del V_T y la k_2 se vieron

afectadas por la duración del escaneo. Después de la inhibición de los P-gp, los valores de K_1 y V_T aumentaron en todas las regiones cerebrales y en todas las duraciones de exploración, mientras que los valores de k_2 disminuyeron en todas las duraciones de exploración excepto en la duración de 91-min. El V_T fue el parámetro mas afectado por la inhibición. En consonancia con los estudios previamente realizados en otras especies animales, el radiotrazador fue rápidamente metabolizado. Por ello, nuestros resultados sugieren el uso de escaneos cortos para evitar la interferencia de radiometabolitos en la cuantificación cinética. En resumen, nuestro estudio apoya el uso de la K_1 , calculada utilizando 1-TCM y escaneos cortos (de 10- a 30-min), como el parámetro mas apropiado para la medida de la función de los P-gp en la BHE en primates.

En el **Capítulo 6** utilizamos los datos obtenidos en los **Capítulos 4 y 5** para realizar una comparación directa entre [^{18}F]MC225 y (R)-[^{11}C]Verapamil. Con este fin, los datos de los estudio PET de 30-min de duración de ambos radiotrazadores fueron ajustados al 1-TCM siguiendo las recomendaciones previamente publicadas (10,11). Los parámetros cinéticos K_1 , V_T y k_2 obtenidos de los escáneres en condiciones basales y en condiciones de inhibición fueron comparados entre los radiotrazadores. Se analizaron también las diferencias regionales en la captación del radiotrazador y se propusieron modelos cinéticos simplificados para la cuantificación de la función de los P-gp. En condiciones basales, los valores de V_T de [^{18}F]MC225 fueron mayores y los valores de k_2 fueron menores que aquellos obtenidos con (R)-[^{11}C]Verapamil. Los valores de K_1 no fueron significativamente diferentes entre los radiotrazadores. Después de la inhibición de los P-gp, los valores de K_1 y k_2 de (R)-[^{11}C]Verapamil fueron más altos que aquellos obtenidos con [^{18}F]MC225, mientras que los valores de V_T de ambos radiotrazadores no fueron significativamente diferentes. La inhibición de la función de los P-gp aumentó los valores de K_1 y V_T en ambos radiotrazadores, y en el caso de (R)-[^{11}C]Verapamil, la inhibición también causó una disminución de los valores de k_2 . Este estudio comparativo sugiere que los valores de V_T de ambos radiotrazadores son igualmente sensibles para medir disminuciones de la función de los P-gp en la BHE en primates. Con respecto a (R)-[^{11}C]Verapamil, este estudio sugiere que el V_T debe ser un parámetro más adecuado que la K_1 para medir la función de los P-gp. Este estudio también mostró diferencias regionales significativas entre los radiotrazadores en condiciones basales, las cuales desaparecieron después de la inhibición. Los resultados de ambos radiotrazadores indicaron que las regiones subcorticales deben presentar una mayor función de los P-gp que el córtex frontal y el cerebelo. Como modelo cinético simplificado, el estudio muestra que la pendiente positiva extraída de las SUV-TAC (curvas de actividad (SUV) con respecto al tiempo) correlaciona positivamente con los valores de K_1 y V_T en ambos radiotrazadores. Además, los resultados confirman que [^{18}F]MC225 presenta una mayor V_T en condiciones basales que (R)-[^{11}C]Verapamil, lo cual, como se ha mencionado previamente, puede que permita la

cuantificación de incrementos en la función de los P-gp, aunque estudios adicionales son necesarios para verificar esta habilidad.

La actual síntesis de [^{18}F]MC225 implica dos pasos. El objetivo del **Capítulo 7** fue optimizar la producción de [^{18}F]MC225, la cual actualmente implica la destilación de [^{18}F]fluoroethylbromide en un paso intermedio. Por ello, fue desarrollado un nuevo precursor que permitiese la producción de [^{18}F]MC225 a través de una radiofluorinización directa. Se produjo con éxito un precursor que contiene un grupo mesilato (precursor mesilato), sin embargo, este compuesto resultó no ser estable debido a la adición del buen grupo saliente (mesilato). Finalmente, se produjo una sal del precursor mesilato con lo que se consiguió mejorar la estabilidad del compuesto durante más tiempo. Sin embargo, este precursor mesilato presentó varias impurezas, y aunque se llevaron a cabo diferentes métodos de purificación, no se consiguió mejorar su pureza. Sin embargo, [^{18}F]MC225 se consiguió producir con éxito mediante la síntesis simplificada (de un solo paso) y el nuevo precursor, y la radiosíntesis fue automatizada en un módulo de radiosíntesis (IBA Synthera).

Finalmente, en el **Capítulo 8** se utilizó [^{18}F]MC225 para evaluar el efecto de un nuevo inductor de los P-gp (MC111) sobre la función de los transportadores de P-gp. Una disminución en la función de los P-gp en la BHE puede incrementar la concentración de compuestos neurotóxicos dentro del cerebro, lo cual puede estar relacionado con la aparición y la progresión de enfermedades neurodegenerativas. Actualmente, los inductores de los P-gp son considerados como potenciales candidatos para el tratamiento de estas enfermedades (12,13). MC111 fue identificado como un inductor de la función de los transportadores de P-gp y BCRP (por sus siglas en inglés, Breast Cancer Resistance Protein) *in vitro*, se considera que esta doble actividad inductora puede ser beneficiosa para el tratamiento de tales afecciones neurológicas. Por ello, nuestro estudio se centra en verificar el efecto inductivo de MC111 *in vivo* utilizando la imagen PET. Para ello, ratas sanas fueron inyectadas como solución vehículo (grupo control), con 4.5 mg/kg de MC111 (grupo de dosis baja) o con 6 mg/kg de MC111 (grupo de alta dosis) un día antes del escáner PET con [^{18}F]MC225. Después del escáner PET, los animales fueron sacrificados y el tejido cerebral fue extraído para evaluar la expresión de los P-gp utilizando western blot. Nuestros resultados mostraron que la administración de 6 mg/kg de MC111 disminuyen significativamente los valores de K_1 y V_T de [^{18}F]MC225 en los animales tratados en comparación con las ratas controles, lo que indica un aumento en la función de los P-gp debido a la administración de MC111. Sin embargo, los resultados del western blot no encontraron un incremento en los niveles de expresión de los P-gp en las ratas tratadas. Varios mecanismos pueden inducir la expresión de los P-gp en la superficie de las células. Dado que los niveles de expresión de los P-gp en el cerebro no aumentaron después de la administración de MC111, se sugiere que MC111 debe aumentar la expresión de los P-gp en la superficie de las células

endoteliales del cerebro reubicando la proteína P-gp intracelular en la membrana plasmática (14,15). En general, la disminución de los valores de K_1 y V_T de [^{18}F]MC225 indican que se ha producido un incremento en la función de los P-gp en la BHE de las ratas tratadas. Por lo tanto, los resultados también verifican la habilidad de [^{18}F]MC225 para medir aumentos en la función de los P-gp en las ratas.

Podemos concluir que los resultados presentados en esta tesis proporcionan suficiente información para respaldar la translación clínica de [^{18}F]MC225. [^{18}F]MC225 ha mostrado una farmacocinética más adecuada que el radiotrazador de referencia para medir la función de los P-gp, el (R)-[^{11}C]verapamil. La mayor ventaja de [^{18}F]MC225 son sus elevados valores de V_T y su habilidad para medir incrementos en la función de los P-gp. [^{18}F]MC225 tiene el potencial de convertirse en el primer radiotrazador marcado con flúor capaz de medir tanto disminuciones como aumentos en la función de los P-gp en la BHE.

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Chapter 13

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About the author

Lara Garcia Varela was born in Lugo (Galicia, Spain) on 24th May 1992. She had lived in A Coruña (Galicia, Spain) until she started her Pharmacy Bachelor and Master-Degree at the University of Santiago de Compostela (Galicia, Spain).

In 2015, Lara entered the Nuclear Imaging Group at the University Hospital of Santiago de Compostela where she worked on a research project about the effect of diazepam on glucose metabolism under the supervision of Prof. Alvaro Ruibal and Dr. Pablo Aguiar. Also, during her ungraduated studies, she moved to Budapest (Hungary) to do an internship in a community pharmacy and she attended the University Hospital Fundación Jimenez Díaz (Madrid, Spain) for an internship in the Hospital Pharmacy Department. Lara obtained her Pharmacy Bachelor and Master Degree diploma in 2016 with a thesis entitled “Effect of Diazepam on Cerebral Glucose Metabolism in Preclinical PET Studies”. The same year, her Master's degree thesis was awarded as an oral presentation at the “International Student Congress of (bio)Medical Science” (ISCOMS) in Groningen, the Netherlands. Moreover, Lara was awarded a two-week internship (ISCOMS Research Fellowship) in the Department of Nuclear Medicine and Molecular Imaging at the University Medical Center of Groningen (UMCG).

After her graduation, Lara worked as a technical assistant of MicroPET and MicroSPECT in the Molecular Imaging Group at the University Hospital of Santiago de Compostela.

In November 2016, she received a Research Voucher from the Abel Tasman Talent Program to do a short research internship in the Department of Nuclear Medicine and Molecular Imaging at UMCG.

In 2017, she was accepted as a PhD candidate by the Abel Tasman Talent Program, and she started her PhD in the Nuclear Medicine and Molecular Imaging Department under the supervision of Prof. Philip Elsinga and Dr. Gert Luurtsema at the UMCG. During her PhD, she investigated the function of the P-glycoprotein (P-gp) transporter located at the Blood-Brain Barrier under different conditions and in different animal species using Positron Emission Tomography (PET) and the novel P-gp PET tracer [¹⁸F]MC225.

List of Publications

- (1) Silva-Rodríguez, J.*; **García-Varela, L.***; López-Arias, E.; Domínguez-Prado, I.; Cortés, J.; Pardo-Montero, J.; Fernández-Ferreiro, A.; Ruibal, Á.; Sobrino, T.; Aguiar, P. **Impact of Benzodiazepines on Brain FDG-PET Quantification after Single-Dose and Chronic Administration in Rats.** *Nucl. Med. Biol.* **2016**, *43* (12), 827–834. <https://doi.org/10.1016/j.nucmedbio.2016.09.001>.
- (2) Fernández-Ferreiro, A.; Silva-Rodríguez, J.; Otero-Espinar, F. J.; González-Barcia, M.; Lamas, M. J.; Ruibal, A.; Luaces-Rodríguez, A.; Vieites-Prado, A.; Sobrino, T.; Herranz, M.; **García-Varela, L.**; Blanco-Mendez, J.; Gil-Martínez, M.; Pardo, M.; Moscoso, A.; Medín-Aguerre, S.; Pardo-Montero, J.; Aguiar, P. **Positron Emission Tomography for the Development and Characterization of Corneal Permanence of Ophthalmic Pharmaceutical Formulations.** *Invest. Ophthalmol. Vis. Sci.* **2017**, *58* (2), 772–780. <https://doi.org/10.1167/iovs.16-20766>.
- (3) Fernández-Ferreiro, A.; Luaces-Rodríguez, A.; Aguiar, P.; Pardo-Montero, J.; González-Barcia, M.; **García-Varela, L.**; Herranz, M.; Silva-Rodríguez, J.; Gil-Martínez, M.; Bermúdez, M. A.; Vieites-Prado, A.; Blanco-Méndez, J.; Lamas, M. J.; Gómez-Ulla, F.; Ruibal, Á.; Otero-Espinar, F. J.; González, F. **Preclinical PET Study of Intravitreal Injections.** *Invest. Ophthalmol. Vis. Sci.* **2017**, *58* (7), 2843–2851. <https://doi.org/10.1167/iovs.17-21812>.
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- (5) **García-Varela, L.**; Váñez García, D.; Rodríguez-Pérez, M.; van Waarde, A.; Sijbesma, J. W. A.; Schildt, A.; Kwizera, C.; Aguiar, P.; Sobrino, T.; Dierckx, R. A. J. O.; Elsinga, P. H.; Luurtsema, G. **Test–Retest Repeatability of [¹⁸F]MC225-PET in Rodents: A Tracer for Imaging of P-gp Function.** *ACS Chem. Neurosci.* **2020**, *11* (4), 648–658. <https://doi.org/10.1021/acscchemneuro.9b00682>.
- (6) **García-Varela, L.**; Arif, W. M.; Váñez García, D.; Kakiuchi, T.; Ohba, H.; Harada, N.; Tago, T.; Elsinga, P. H.; Tsukada, H.; Colabufo, N. A.; Dierckx, R. A. J. O.; van Waarde, A.; Toyohara, J.; Boellaard, R.; Luurtsema, G. **Pharmacokinetic Modeling of [¹⁸F]MC225 for Quantification of the P-Glycoprotein Function at the Blood–Brain Barrier in Non-**

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- (9) **García-Varela, L.**; García, D. V.; Kakiuchi, T.; Ohba, H.; Nishiyama, S.; Tago, T.; Elsinga, P. H.; Tsukada, H.; Colabufo, N. A.; Dierckx, R. A. J. O.; van Waarde, A.; Toyohara, J.; Boellaard, R.; Luurtsema, G. **Pharmacokinetic Modeling of (R)-[¹¹C]Verapamil to Measure the P-Glycoprotein Function in Nonhuman Primates.** *Mol. Pharm.* **2021**, *18* (1), 416–428. <https://doi.org/10.1021/acs.molpharmaceut.0c01014>.
- (10) **García-Varela, L.**; Vállez García, D.; Aguiar, P.; Kakiuchi, T.; Ohba, H.; Harada, N.; Nishiyama, S.; Tago, T.; Elsinga, P. H.; Tsukada, H.; Colabufo, N. A.; Dierckx, R. A. J. O.; van Waarde, A.; Toyohara, J.; Boellaard, R.; Luurtsema, G. **Head-to-head comparison of (R)-[¹¹C]verapamil and [¹⁸F]MC225 in non-human primates, tracers for measuring P-glycoprotein function.** *Eur. J. Nucl. Med. Mol. Imaging* **2021**. <https://doi.org/10.1007/s00259-021-05411-2>.
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