
Internship thesis

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Imaging of the H₄R – Preclinical evaluation of [¹¹C]JNJ-39594906 in rat brain

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<tbody>
<tr>
<td>ATP</td>
<td>Attached proton test</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DIPA</td>
<td>Di-isopropylamine</td>
</tr>
<tr>
<td>DIPEA</td>
<td>Di-isopropylethylamine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximum inhibitory concentration</td>
</tr>
<tr>
<td>EOS</td>
<td>End of synthesis</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HDC</td>
<td>Histidine decarboxylase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>H&lt;sub&gt;1&lt;/sub&gt;R</td>
<td>Histamine H&lt;sub&gt;1&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;R</td>
<td>Histamine H&lt;sub&gt;2&lt;/sub&gt; receptor</td>
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<td>H&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Histamine H&lt;sub&gt;3&lt;/sub&gt; receptor</td>
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<td>H&lt;sub&gt;4&lt;/sub&gt;R</td>
<td>Histamine H&lt;sub&gt;4&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>INMiND</td>
<td>Imaging of Neuroinflammation in Neurodegenerative Diseases</td>
</tr>
<tr>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MeI</td>
<td>Methyliodide</td>
</tr>
<tr>
<td>MeOTf</td>
<td>Methyltriflate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post injection</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RCY</td>
<td>Radio chemical yield</td>
</tr>
<tr>
<td>SA</td>
<td>Specific activity</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSPO</td>
<td>Translocator protein</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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Abstract
This thesis describes the research for a new radioligand targeting the histamine H₄ receptor (H₄R). This receptor is found to be expressed on microglia and to play an important role in the chemotaxis of those macrophages. Microglia can have a neurodegenerative effect on the brain if chronic neuroinflammation is present. These findings caused the search for a possibility to image and quantify H₄R in the healthy and inflamed brain by positron emission tomography (PET). The H₄R antagonist JNJ-39594906 was found to be brain penetrable and has a high affinity and selectivity for H₄R, making it a good candidate for the development of a PET ligand. First the non-radioactive reference compound JNJ-39594906 was synthesized together with the desmethyl precursor for the radiolabelling. Using an in-house developed automated synthesis unit, [¹¹C]JNJ-39594906 was then synthesized by nucleophilic substitution with [¹¹C]MeI or [¹¹C]MeOTf. Using [¹¹C]MeI as methylation agent resulted in 11.7 ± 1.3 conversion and 6.0 ± 0.7% decay corrected radiochemical yield (RCY). When [¹¹C]MeOTf was used, a conversion of 21.5 ± 1.0% and an RCY 8.7 ± 1.4% could be achieved. When sufficient amounts of radioactivity were produced preclinical animal studies were conducted. At first in vitro autoradiography studies revealed high nonspecific binding to brain tissue. Ex vivo metabolite analyses and biodistribution studies revealed a fast metabolism in the periphery, and low amounts of intact [¹¹C]JNJ-39594906 in plasma and especially the brain. Therewith [¹¹C]JNJ-39594906 is assessed as not suitable to image H₄R by PET.
1. Introduction
INMiND is a project of the European Union’s Seventh Framework Programme (FP7/2007-2013) and aims the Imaging of Neuroinflammation in Neurodegenerative Diseases. Its goal is to carry out “collaborative research on molecular mechanisms that link neuroinflammation with neurodegeneration in order to identify novel biological targets for activated microglia” (INMiND 2007). One of the partners is the Radio Nuclide Center (Department of Radiology and Nuclear Medicine) at the Vrije Universiteit Medical Center together with its spin-off company Cyclotron BV. Their task is to develop new radioligands for Positron Emission Tomography (PET) targeting activated microglia, which can lead to advanced diagnostic imaging or even new pharmaceuticals to treat neuroinflammation.

The histamine H₄ receptor is of interest for the pharmaceutical research and development, because of its involvement in neuroinflammation. The receptor is expressed on neuronal cells as well as microglia in the central nervous system (CNS). Microglia are the resident macrophages in the brain and spinal cord, and act in neuroinflammatory processes. Therefore the H₄R and microglia are considered to be an appropriate target for visualization and quantification of neuroinflammation in PET. A possible radioligand for the imaging of H₄R could be [¹¹C]JNJ-39594906, because of its high affinity for H₄R and the ability to penetrate the blood brain barrier (BBB).

The results achieved in the synthesis and preclinical investigations of [¹¹C]JNJ-39594906 as a radioligand for the H₄R will be described in this thesis.

The following chapters describe the fundamental principles, the aim of this project and the methods that were used for synthesis, radiosynthesis and animal studies. After that the results are presented en discussed. In the end there is a conclusion and a future perspective on the imaging of H₄R.
2. Fundamentals

2.1 Histamine
Histamine is an endogenous organic compound in the body, which regulates immune responses, acidity of the gut and acts as a neurotransmitter (Haas, Sergeeva en Selbach 2008). Histamine is a product of the de-carboxylation of the essential amino acid L-histidine, which is catalyzed by the enzyme Histidine decarboxylase (HDC), see figure 1.

![Image of L-Histidine and Histamine with Histidine decarboxylase reaction](image)

Figure 1: The de-carboxylation of L-histidine to histamine.

After its biosynthesis, histamine can be released directly, or stored in mast cells. If these mast cells are stimulated, histamine is released and that causes symptoms of an allergic reaction which include itch, swelling and flushing (Shahid, et al. 2009).

2.2 Histamine receptors
There are four different receptor subtypes discovered which interact with histamine: H1R, H2R, H3R and H4R. These receptors belong to a super family of receptors that are called G-protein coupled receptors. This super family includes approximately 1000 different receptors and around 30% of all current drugs target these GPCRs (Jacoby, et al. 2006).

GPCR are often called 7 transmembrane domain receptors, because their protein α-helixes pass through the cell membrane seven times. Four of these helixes form a cavity in the membrane. When activated or deactivated by a ligand, the conformation of the protein changes, resulting in intercellular signaling (Katritch, Cherezov en Stevens 2013).

H1R are found in different mammalian tissues. The receptors are present in various cells, including those of the CNS, airway and vascular smooth muscle cells. In the periphery, typical H1R responses are redness, itching and swelling, classical symptoms of an allergic reaction (Shahid, et al. 2009). H1R is also expressed in most of the brain region of non-human primates and humans. Deficiencies in H1R neurotransmission result in disturbance of circadian rhythm, feeding behavior and energy metabolism. It’s also found to be involved in neuroinflammation.

H2R are found mostly in the stomach area, but also in numerous other cell types such as heart and smooth-muscle cells. The H2R plays an important role in the gastric acid secretion (Hill, et al. 1997).
H₃R are mainly expressed in histaminergic neurons of the CNS, and have a low expression in peripheral neurons. The H₃R regulates the release of histamine and other neurotransmitters such as dopamine, acetylcholine, noradrenalin and serotonin (Schlicker en Kathmann 1998) and is often connected with stimulant and nootropic effects.

H₄R have a high expression in intestine tissue, the spleen, bone marrow and peripheral hematopoietic cells. By regulating the chemotaxis of mast cells the H₄R plays a key role in inflammation and immunity regulations (Shahid, et al. 2009). Neural expression of active H₄R has been shown in multiple brain regions of the mouse and human (Connelly, et al. 2009). The H₄R are confirmed to play a role in neuroinflammation, because it is also expressed on microglia, the resident macrophages of the brain and spinal cord (Ferreira, et al. 2012). The binding pocket of the H₄R is shown in figure 2, together with a ligand inside the binding pocket.

2.3 Microglia and neuroinflammation

Inflammation in the periphery is a complex response to harmful pathogens. It is considered a self-defense mechanism to protect an organism to exogenous stimuli. The specification neuroinflammation is a relatively new term in particular for inflammation in the CNS. There, inflammation is distinctive because it does not reproduce the common characteristics of inflammation in the periphery (Streit, Mrak en Griffin 2004). A microglia is shown in figure 3.

Acute neuroinflammation is the endogenous response to injury in the nervous system and chronic neuroinflammation is strongly related to neurodegenerative diseases. It is considered to cause neuronal loss in Alzheimer’s disease, Multiple Sclerosis, Parkinson’s disease and Huntington’s disease.

Unlike the periphery, the brain has no white blood cells. Microglia are considered to be the macrophages of the CNS, controlling the brain for infections and injury. If infections or injury take place, microglia become activated, migrate to the site and remove damaged cells by phagocytosis (Dheen, Kaur en Ling 2007). Thus microglia are per se important for the brain immune system. However, in chronic neuroinflammation the activation of microglia can trigger cascades of neurotoxics which degenerate tissue progressively (Amor, et al. 2013). This difference between activated microglia that protect the brain, and over activated ones which cause harm is a big and important research topic.
2.4 Histamine H₄ receptor on Microglia
Microglia are found to express all four different histamine receptors. Histamine plays an active role in chemotaxis of most of the cells expressing histamine receptors. This was also found for microglia, but the chemotaxis was specific for H₄R (Ferreira, et al. 2012). In this study, multiple tests were conducted on microglia that migrated in a scratch wound assay. At first, incubation with histamine induced the migration of microglia. Coincubations with H₁R-, H₂R-, and H₃R antagonists did not inhibit this movement. However, coincubating with histamine and JNJ-7777120, an H₄R antagonist, completely blocked the microglia migration. In this way it was shown that the chemotaxis of microglia works via H₄R activation.

2.5 Positron Emission Tomography
PET is a technology designed to study the way molecules are divided inside organisms. It produces a three dimensional image showing the concentrations of compounds, based on radioisotopes decaying and sending out positrons. Those short living isotopes are usually coupled to biologically active molecules. When injected intravenously, such a radioactive compound will be transported to its biological destination. At one point in time, the radioisotope will decay and primarily send out a positron. This positron will counter an electron, which acts as its antiparticle. In the moment of its collision they both annihilate, creating a pair of photons. These photons move in opposite directions and can be detected by the circularly arranged detectors of a PET scanner, as shown in figure 4. In this way, place and time of the origin of the photon can be determined, and so the location of the radio labeled molecule is known. From all the detected incidents of decay, a tridimensional image can be reconstructed.

2.6 PET isotopes and their application
Various isotopes are used in PET-scans, varying in half lives and their atom characteristics. The most used are short living nuclides like carbon-11, nitrogen-13, oxygen -15 and fluorine-18. Besides those, longer living isotopes are used, like bromine-76 and iodine-124. Also radioactive metals can be applied in PET studies such as the positron emitting isotopes of copper, gallium and technetium.

The shorter living isotopes can be created by a cyclotron. Such a cyclotron is used to accelerate small particles like protons, electrons or ions. Under influence of a high frequency alternating voltage between two D-shaped electrodes and a static magnetic field, the particles speed up in a circular way. When the particles have enough velocity, they will be redirected into a chamber providing corresponding atoms or molecules, the so called target. The collision of a particle or ion with an atom results into a nuclear reaction, creating the radioisotope.

Carbon-11 is a widely used PET-isotope with a short half-life of 20.38 minutes. Carbon-11 can be created by the collision between protons and nitrogen-14 molecules ($^{14}$N$_₂$) in gas phase. In this case, the collided...
nitrogen atom emits an α-particle. This reduces the atom mass by 4, and the number of protons by 2, leading to a carbon-11 atom. When 2% of oxygen or 5%-10% of hydrogen is added to the nitrogen target gas, $^{11}\text{CO}_2$ or $^{11}\text{CH}_4$ is created. These molecules are important precursors in labeling reactions (Schubiger, Friebe en Lehmann 2007).

Radiosyntheses are carried out under special conditions as they require protection of the operator from emission of energetic particles or rays. Therefore the radioactive methane or the carbon dioxide are transported to the special designed cabinets with leaden doors called “hot cells”, see figure 5. Computer assisted modules installed in those hot cells facilitate semi or full automated radiosynthetic and preparation procedures.

The $^{11}\text{CO}_2$ can be used directly to react with primary amines or organometallic compounds (Allard, et al. 2008). After that, $[^{11}\text{C}]$methyl iodide is the most used radiolabeling agent for the alkylation of various nucleophilic compounds. In Figure 6, more pathways are shown for useful carbon-11 compounds in radio labeling (Schubiger, Friebe en Lehmann 2007).

Because carbon-11 has a half-life of 20.38 minutes, synthesis and preparation of carbon-11 labeled compounds has to be performed as rapid as possible, with introduction of the radionuclide at the latest possible time point in multi-step syntheses. But since there is a high excess of precursor (millimolar range) and much less radioactive carbon-11 labeling agent (nanomolar range)
present in a reaction mixture, such a synthesis possesses the kinetics of a pseudo-first order reaction, even though it follows a $S_N2$ mechanism. The chance that a radioactive methyl iodide molecule reacts with a precursor molecule (in a high and quasi constant concentration) is much higher. This can decrease the reaction times of hours under normal organic synthesis conditions to a few minutes. However, the low concentration of the radionuclide places special demand on the purity of starting materials and reaction media to avoid competitive reactions with impurities (Dolle, et al. 2008).

2.7 Radioligands for PET imaging of H4R

A ligand which acts as a receptor agonist is a compound that triggers a receptor protein signal in the same way the endogenous compound does. Thereby the ligand mimics, or can even show an increased effect in comparison to the endogenous compound. An antagonist does not create a biological response in a receptor by itself, but can stabilize the activated state of the receptor or inhibit the binding of an agonist. The binding potential of a ligand is usually displayed in half maximal inhibitory concentration ($IC_{50}$). Determined in vitro, it is the concentration of a solution necessary to incubate 50% of a receptor. The affinity of a compound to a receptor is usually described by its $K_i$ value. The $K_i$ is the inhibition constant of a drug and determined in competition with another receptor specific compound with known receptor affinity. It is then the equilibrium between unbound and bound ligand.

For the first three histamine receptors all various radioligands have been developed and investigated, targeting specifically one of the subtypes (Funke, Vugts, et al. 2013) (Funke, Janssen, et al. 2014). When the H4R was discovered, a high throughput screening was performed and resulted in the highly potent and selective H4R antagonist JNJ-7777120 (Jablonowski, et al. 2003). The indole based compound showed high affinity to human H4R with a $K_i$ of 4 nM and is shown in figure 7A. For PET ligand development the carbon-11 labelled analogue was synthesized by methylation of the corresponding free piperazine (Smits, et al. 2009). By using PET the radioligand was shown to enter the brain rapidly and it turned out to be metabolic stable (Funke, Smits, et al. 2014). Making it a potential PET tracer for imaging of H4R expression in the brain, it is currently investigated in animal models developing neuroinflammation.

The second development of a H4R selective PET ligand was based on the quinazolineVUF-10558 (Figure 7B) with an $K_i$ on human H4R of 4 nM. However, this radiotracer was not viable since it did not enter via the BBB in significant amounts and stayed in the periphery over a period of 60 min, as observed in biodistribution studies (Smits, et al. 2009).

A novel, by Johnson & Johnson developed H4R antagonist is the pyrimidine JNJ-39594906, shown in figure 8B. The corresponding patent describes the synthesis and in vitro studies of various structures that contain a 2-aminopyrimidine moiety (Cai, et al. 2013).
The structural related H₄R antagonist JNJ-39758979 (figure 8A) revealed a very high affinity to human H₄R (Engelhardt, et al. 2013) and showed an excellent safety profile in rats and monkeys (Thurmond, et al. 2014). It has been tested in three different clinical studies, whereof the first was a proof of concept of the compound to investigate if JNJ-39758979 prevented itching induced by histamine. The trial was completed but no results have been published so far (Clinicaltrials.gov1 2010). In the second safety and pharmacokinetic study, JNJ-39758979 was investigated in healthy volunteers; whereof no data are currently available (Clinicaltrials.gov2 2010). In the following a phase II study was conducted on volunteers afflicted with persistent asthma. This study ended in May 2013 and so far no results have been reported as well (Clinicaltrials.gov3 2013).

Nevertheless, JNJ-39758979 has been demonstrated to enter the rat brain (Savall 2013), which makes the compound an interesting chemical lead for the development of a PET tracer for neuroimaging of the H₄R. JNJ-39594906 is the N-methylated analogue of JNJ-39758979 and revealed comparable Ki values. The Ki value for human H₄R of JNJ-39758979 was 20 nM and 7 nM for JNJ-39594906 (Cai, et al. 2013). Another study reports 12 nM for JNJ-39758979 (Wersinger 2013). Based on the promising in vitro properties and the possibility to synthesize its carbon-11 labeled analogue by [11C]methylation, this structure is investigated as potential H₄R PET tracer, as described in the following chapters.

2.8 Preclinical Studies

Before pharmaceuticals are tested in humans (clinical trials), preclinical trials are performed. These investigations are usually performed on cells expressing the target of interest, tissue samples (in vitro) or in animals (in vivo and ex vivo). In the research for a neuroreceptor-ligand like [11C]JNJ-39594906 several preclinical studies are performed to see if the compound could be a viable PET tracer for neuroimaging. At first, specific and selective binding to the target will be determined by in vitro autoradiography on rat brain slices. If there is specific and target-selective binding observed, PET studies can be performed in rats. Also radioactive metabolite studies will be performed to know what percentage of the radioactivity is related to parent compound. In the end biodistribution studies in rats will give a representation of the distribution of the radioligand in tissues and organs.

2.8.1 In vitro autoradiography

In vitro investigations are a way to study the binding and kinetic properties of compounds on cells or parts of a dissected animal. For the binding efficiency of [11C]JNJ-39594906, slices from snap frozen rat brains are used. Such slices are mounted on object plates, and incubated with the radioligand. These slices with bound radioactivity are then placed under a film or a storage phosphor slide to create a two dimensional image of the distribution of the desired radiotracer.
For example in figure 9, the distribution of the translocator protein (TSPO) tracer $[^{11}\text{C}]$PK-11195 in a rat brain is shown. The upper image shows two brain halves. Before section of the animal, the left brain half was injected with lipopolysaccharide (LPS), an induced acute neuroinflammation model. The other half of the brain is used as healthy control. Increased uptake of $[^{11}\text{C}]$PK-11195 is clearly shown in the left half of the first rat brain. This makes PK-11195 a potential tracer for neuroinflammation. In the second rat brain, the slices where preincubated with GE-180, a different neuroinflammation tracer expect to bind on the same receptor. Because there is no radioligand binding in the blocked brains, the selectivity of $[^{11}\text{C}]$PK-11195 is proven (Dickens, Vainio en Johansson 2014). Nevertheless, the overall green color indicates non-specific binding, means binding to various proteins of the brain tissue. For a radiotracer, the least possible amount of nonspecific binding is wanted, because it complicates clear localisation and quantification of the real receptor binding.

For the autoradiography of $[^{11}\text{C}]$JNJ-39594906, binding to healthy brains, binding in case of neuroinflammation using the LPS model, and different blocking studies will be carried out. Blocking with different concentrations of competing H$_2$R ligands give an indication of the binding potential of $[^{11}\text{C}]$JNJ-39594906.

2.8.2 In vivo animal PET

The ultimate goal is to create a PET-ligand which could be used as a tracer in humans. Clinical trials will only be approved if the PET-ligand is found to be safe in preclinical animal studies. Although the results of PET in rodents are not fully comparable to PET studies in humans, they can give a good indication of physiological and binding properties of the radiotracer in a living organism. The corresponding technique of PET is described above in section 2.5 and 2.6.

An example is given in figure 10, showing an MRI and three PET images of rat skulls. At first by means of MRI, the ROIs, in this case the rat brain regions are determined. The following two PET-images show $[^{11}\text{C}]$phenytoin uptake in rat brain whereof the first PET-image depicts a rat brain with preadministration of the blocking substance tariquidar. The last image is an overlay of the MRI image on a PET image, to combine the precise anatomical information with the functional information from the PET-scan (Verbeek, et al. 2012).
The binding of the radioligand can also be unspecific. This means that it is binding to other sites than the wanted receptor. This can be detected by administration of a 100 fold of “cold” ligand to the test subject. This will block all the H4 receptors and further specific binding sites present. When eventually administrating the radioligand, all bound radioactivity will be nonspecific. These scans can be deducted from a normal scan, resulting in a scan without nonspecific binding.

The same models as applied in vitro will be used for the determination of $^{11}$CJNJ-39594906 uptake in brain in vivo. Healthy rats will be used for determination of total binding in brain, and blocking with cold JNJ-39594906 or other H4R antagonists like JNJ7777120 or Thioperamide. If these results are satisfactory, rats with neuroinflammation using LPS injection in the brain can be scanned with $^{11}$CJNJ-39594906, to see if H4R expression increases with neuroinflammation.

2.8.3 Biodistribution

With biodistribution studies it is possible to determine the $^{11}$CJNJ-39594906 concentration in different organs and tissues. The animals are sacrificed and dissected; the organs and tissues are counted for radioactivity. Primarily high radioactivity is wanted in the brain to make $^{11}$CJNJ-39594906 a viable neuroinflammation tracer for PET. High radioactivity in the bladder or liver can indicate fast clearance and metabolism.

For example in figure 11, two graphs are shown of the biodistribution of $^{11}$Clanquidar, a P-gp substrate. P-gp is an active transporter in the BBB, and differences in expressions may play a role in brain disorders. In this study 20 MBq of $^{11}$Clanquidar was injected in 16 rats. At 5, 15, 30 and 60 minutes 4 rats were sacrificed, respectively. Multiple organs, tissues (Figure 10 A) and brain regions (Figure 10B) were dissected, weighed, and counted for activity. The results show a very fast clearance of $^{11}$Clanquidar from the blood, and high uptake in the lungs. The uptake in the investigated brain regions was low and nearly identical, except for the olfactory bulb. The conclusion was that lanquidar would be a P-gp inhibitor instead of a P-gp substrate (Luurtsema, et al. 2009).

2.8.4 Metabolite analysis

Radioactive tracers can be like any other pharmaceutical metabolized by enzymes in the rats. Therefore metabolite analysis are carried out to determine if metabolism takes place and in what rate. In case of radiotracers for neuroimaging especially the metabolism or metabolic content in the brain and in the transport medium plasma is of interest. The radioactive metabolites are unwanted, since they can produce a false positive or high background in PET-scans and biodistribution. If $^{11}$CJNJ-39594906 will be rapidly metabolized in brains, the tracer will not be useful for specific H4R imaging and therewith of
If moderate metabolism takes place in plasma, the radioligand might still be useful since mostly polar metabolites will not enter the brain due to the BBB. If only small amounts of lipophilic metabolites are present in the brain, the tracer might still be useful, since small amounts of radioactive metabolites can be corrected off and might not influence radioligand binding to the target.

For example, the metabolite analysis of \([^{11}C]\)phenytoin was carried out in the following way (Verbeek, et al. 2012). For this study, Sprague – Dawley rats were injected with around 200 MBq of \([^{11}C]\)phenytoin and blood samples were taken at certain time-points. At two time points the rats were euthanized and the brain was collected. The blood samples where and plasma was collected. The plasma was loaded on tC-18 Sep-Pak and washed with water. This fraction was collected as the polar fraction, containing the hydrophilic radiometabolites. The Sep-Pak was then eluted with methanol, containing the non-polar fraction of radiometabolites and parent compound. All fractions were analyzed on HPLC. The brain was homogenized by ultrasonic homogenization in water, under ice cooling. The homogenized mixture was centrifuged and the supernatant was loaded on a tC-18 Sep-Pak. Washing with water was again followed by extraction with methanol, and polar as well as non-polar fractions where analyzed with HPLC.

Metabolism in plasma was fast with only 19% of intact tracer after 45 minutes p.i., shown in table 1. In the brain, 83% of tracer was still intact after 45 minutes. Also the two radioactive metabolites that were found in blood, were not observed in the brain, indicating that no metabolites entered the brain via the BBB (Verbeek, et al. 2012). Comparable results for the metabolism in brain would be appropriate for \([^{11}C]\)JNJ-39594906, however a slower metabolism in plasma would be desirable.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Plasma (%)</th>
<th>Brain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>60 ± 7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>50 ± 16</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>38 ± 3</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>28 ± 2</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>30</td>
<td>32 ± 18</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>19 ± 8</td>
<td>83 ± 5</td>
</tr>
</tbody>
</table>

Table 1: Percentage of intact \([^{11}C]\)phenytoin at different time points after injection (N = 2)
3. Aim
The aim for this project was to develop a radioligand for PET imaging of H₄R in the brain, based on the potent and H₄R selective antagonist JNJ-39594906. Its carbon-11 labeled analogue was already synthesized successfully. However, the amount of pure radiotracer at the end of the synthesis has to be improved to make it available for comprehensive preclinical investigations.

If suitable amounts of pure [¹¹C]JNJ-39594906 can be provided, *in vitro* autoradiography binding studies on rat brain material can be carried out.

If these *in vitro* results showed effective and selective binding to H₄R, radioactive metabolite analysis, biodistribution and PET imaging studies in rats can be performed.
4. Experimental

4.1 General

Chemicals are obtained from commercial sources and used without further purification. Solvents are purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) and Biosolve (Valkenswaard, the Netherlands) and used directly, unless stated otherwise. Reactions are performed at room temperature unless stated otherwise. Reactions are monitored by thin layer chromatography (TLC) on pre-coated silica 60 F254 aluminum plates (Merck, Darmstadt, Germany). Spots are visualized by UV light and ninhydrin in 1-butanol. Evaporation of solvents is performed under reduced pressure at 40 °C using a rotary evaporator. Column chromatography is performed manually on Silica gel 60 Å (Merck, Darmstadt, Germany) or on a Büchi (Flawil, Switzerland) Sepacore system (comprising a C-620 control unit, a C-660 fraction collector, two C601 gradient pumps and a C640 UV detector) equipped with Büchi Sepacore prepacked flash columns. Nuclear magnetic resonance (NMR) spectroscopy is performed on a Bruker (Billerica, MA, USA) Avance 250 (250.13 MHz for 1H and 62.90 MHz for 13C) with chemical shifts (δ) reported in parts per million (ppm) relative to the solvent (D2O 4.75 1H). Electrospray Ionization High Resolution Mass Spectrometry (ESI-HRMS) is carried out using a Bruker microTOF-Q instrument in positive ion mode (capillary potential of 4500 V). Analytical isocratic high-performance liquid chromatography (HPLC) is performed on a Jasco (Easton, MD, USA) PU-2080Plus station with a ReproSil Gold 120 C18 5µm (200x4.6 mm) column (Dr. Maisch, Ammerbuch-Entringen, Germany), using H2O/MeCN/DIPA (80/20/0.1) as eluent with a flow of 1mL/min, a Jasco UV-2075 Plus UV detector (278 nm) and a NaI radioactivity detector (Raytest, Straubenhardt, Germany). Semipreparative HPLC was performed using a ReproSpher 100 C18-DE, 5 µm (50x8mm) column (Dr. Maisch, Ammerbuch-Entringen, Germany) and using H2O/EtOH /DIPA (85/15/0.1) as method A or H2O/ MeCN /DIPA (85/15/0.1) as method B and an eluent with a flow of 3mL/min. Using UV/Vis detection at 278 nm. Chromatograms are acquired with Raytest GINA Star software (version 5.01). Molecular Dynamics Storage Phosphor screens of 35 x 43 cm (GE Healthcare Europe GmbH, Diegem, Belgium) were used for autoradiography. Readouts of the phosphorscreens were performed on a Typhoon FLA 7000 scanner (GE Healthcare Europe GmbH, Diegem, Belgium). The images were quantified with ImageQuant TL software version 8.1 (GE Healthcare Europe GmbH, Diegem, Belgium). For metabolite analysis and in biodsitributional studies the radioactivity in blood, tissue and organ samples were counted with a 2486 Automatic gamma counter Wallac Wizard 2 3” (Perkin Elmer, Massachusetts, USA) or a 1282 Compugamma CS universal gamma counter (LKB Instruments, Mount Waverley, Australia). For metabolite analysis an UltiMate 3000 Standard LC System (Thermo Fisher Scientific BV, Breda, The Netherlands) was used. The plasma separations were performed on a Gemini 5µm C18 (250 x 10 mm) column (Phenomenex, Torrance, California, USA) using H2O/ MeCN /DIPA (75/25/0.1%) as eluent at 4 mL/min. Fractions were collected on a Foxy Jr. Fraction Collector (Teledyne Isco, Lincoln, Nebraska, USA). Healthy male Wistar rats were obtained from Harlan Netherlands B.V. (Horst, the Netherlands). All animal experiments were performed according to National Institute of Health principles of laboratory animal care and Dutch national law (‘Wet op de proefdieren’ Stb 1985, 336).
4.2 Preparation of the precursor for radiolabeling

A preparation of the desmethyl analogue of JNJ39758979, was performed, with adjustments to improve yield (Cai, et al. 2013). The reaction is shown in scheme 1.

![Scheme 1: Organic synthesis of precursor amine salt 4.](image)

4.2.1. Synthesis of (S)-tert-butyl (1-(2-amino-6-isopropylpyrimidin-4-yl)pyrrolidin-3-yl)carbamate (3)

300 mg of unpure aniline 1 was separated by a büchi column, resulting in 274 mg of pure 1 (1,6 mmol). This was dissolved together with pyrrolidin 2 (372 mg, 2 mmol) and 400 µL pyridine in 4 mL ethanol. This was heated to 90 °C and stirred until there was no aniline 1 observed in the reaction mixture via TLC (4 hours). An orange solution was obtained and removed of solvents in vacuo. The crude (S)-tert-butyl (1-(2-amino-6-isopropylpyrimidin-4-yl)pyrrolidin-3-yl)carbamate 3 was separated from the reaction mixture by flash chromatography (Büchi) with a gradient of dichloromethane and methanol from 100:0 to 70:30 over 30 minutes. The solvent removed in vacuo, to give 490 mg of 3 as a yellow solid (1,5 mmol, 95%). MS (ESI): mass calculated for C16H27N5O2, 321.2; m/z found 322.2 [M+H]+. No NMR data was obtained; the crude product was directly used in the deprotection step.

4.2.2. Synthesis of 4-(3-aminopyrrolidin-1-yl)-6-isopropylpyrimidin-2-amine (4)

The BOC-protected amine 4 was dissolved in 2M HCl in 20mL methanol and stirred at room temperature until complete conversion, as controlled by TLC (21 hours). The solvent was evaporated in vacuo. A light yellow solid was obtained (329 mg, 1,5 mmol, quant). MS (ESI): mass calculated for C11H19N5, 221.2; m/z found 222.2 [M+H]+. Analytical HPLC showed no impurities and the compound had the same retention time.

\[
^1H\text{ NMR (D}_2\text{O): 5.97 (d, 1H, Ar-H), 4.18-3.57 (m, 5H, 5xCH}_2\text{), 2.87-2.66 (m, 1H, R}_3\text{NCH), 2.57-2.31 (m, 1H, R}_3\text{NCH), 2.31-2.04 (m, 1H, R}_3\text{CH}_2\text{), 1.30-1.08 (d, 6H, 2xCH}_3\text{), for spectrum see appendix 1.}
\]
$^13$C-NMR (D$_2$O): 164 (quaternary, aromatic), 161 (quaternary, aromatic), 152 (quaternary, aromatic), 90 (tertiary, aromatic), 57 (tertiary), 56 (tertiary, amine), 42 (secondary, amine), 43 (secondary, amine), 26 (secondary), 17 (2x primary), for spectrum see appendix 2.

4.3 Radiosynthesis of $[^{11}C]$JNJ-39594906
Radiosynthesis tests were carried out in a Good Manufacturing Practice (GMP) applying production lab. The hotcells are equipped with in-house made, fully automated and software assisted synthesis units (Windhorst, et al. 2001). A control scheme for the synthesis units can be found in appendix 3. The reaction for the generation of $[^{11}C]$methyl iodide or $[^{11}C]$methyltriflate are shown in scheme 2, followed by the methylation of precursor amine 4.

Scheme 2: The synthesis of $[^{11}C]$methyl iodide or $[^{11}C]$methyltriflate, followed by the radiosynthesis of $[^{11}C]$JNJ-39594906.

10 GBq of $[^{11}C]$CO$_2$ were produced by a $^{14}$N(p,α)$^{11}$C nuclear reaction in a 0.5% O$_2$/N$_2$ gas mixture. The formed $[^{11}C]$CO$_2$ was transported from the cyclotron to a liquid nitrogen-cooled loop. The frozen $[^{11}C]$CO$_2$ was then sublimated by removal of the liquid nitrogen from the loop and warming with compressed air and the gas transported by a helium flow to the hotcell. In the first reaction vial, the $[^{11}C]$CO$_2$ was trapped and reduced by 0.1 mL of 0.1 M LiAlH$_4$ in THF to $[^{11}C]$lithium methoxide (CH$_3$OLi, Scheme 4). The THF was then evaporated under helium flow at 130 °C. Subsequently 250 µL of 57% hydrogen iodide was added resulting in $[^{11}C]$methyl iodide (MeI). By means of a helium flow, the reactant was distilled at 130 °C to a second reaction vial, passing a sicapent/NaOH column to dry it and trap non-reacted $[^{11}C]$CO$_2$. This second reaction vial already contained 0.5 mg of precursor 4 (2.3 µmol), dissolved in 500 µL dimethylsulfoxide (DMSO) and 10µL of a base (8 to 25 µmol, for individual reaction conditions see table 1). The resulting reaction mixture was heated to 70 to 130 °C for 5 minutes.

To evaporate all volatile radioactive compounds, a 20 mL/min flow of helium was blown trough the reaction mixture for 5 minutes and the volatile components were trapped via the exhaust in a Porapak cartridge. After the degassing, the remaining reaction mixture was diluted with 1.5 mL of H$_2$O. A sample
from the reaction mixture was analyzed on analytical HPLC using the method described above (see chapter 4.1.). The retention time of $[^{11}\text{C}]$JNJ39594906 of 13-14 minutes was confirmed by co-elution of the reaction mixtures with non-radioactive reference compound.

4.4 Complete preparation of formulated $[^{11}\text{C}]$JNJ-39594906

The complete radiosynthetic production of $[^{11}\text{C}]$JNJ-39594906 consists of the synthesis procedure as described in paragraph 4.3, followed by semipreparative HPLC and formulation, to create a >99% pure solution of $[^{11}\text{C}]$JNJ-39594906.

10 to 50 GBq of $[^{11}\text{C}]$CO$_2$ were produced by a $^{14}$N(p,α)$^{11}$C nuclear reaction in a 0.5% O$_2$/N$_2$ gas mixture. The formed $[^{11}\text{C}]$CO$_2$ was transported from the cyclotron to a liquid nitrogen-cooled loop. The frozen $[^{11}\text{C}]$CO$_2$ was then sublimated by removal of the liquid nitrogen from the loop and warming with compressed air, and the gas transported by a helium flow to the hotcell. In the first reaction vial the $[^{11}\text{C}]$CO$_2$ was trapped and reduced by 0.1 mL of 0.1M LiAlH$_4$ in THF to $[^{11}\text{C}]$lithium methoxide, as shown in scheme 4. The THF was evaporated out of the mixture and 250 µL 57% hydrogen iodide was added resulting in $[^{11}\text{C}]$MeI. By means of an helium stream passing a sicapent/NaOH column to dry and trap non-reacted $[^{11}\text{C}]$CO$_2$, the reagent was distilled then in a second reaction vial. Alternatively and to obtain $[^{11}\text{C}]$MeOTf as methylation agent, the $[^{11}\text{C}]$MeI/helium stream passed through a Silvertriflate column at 200 °C.

0.5 mg of free base 4 and 10 µL of DIPEA in 500 µL DMSO were loaded into a reaction vial. The $[^{11}\text{C}]$MeI or $[^{11}\text{C}]$MeOTf was trapped in this solution and reacted for 5 or 3 minutes, respectively at 70 °C to 130 °C. After adding 1.5 mL of water to the reaction mixture, the solution containing the crude product was automatically transferred to a 5 mL injection loop and injected on a semipreparative HPLC. In case of HPLC eluents containing EtOH (methods see chapter 4.1) no further additions were made on the fraction that was collected. However if animal studies would be conducted, a concentrated buffer solution should have been added.

If MeCN was used as organic solvent for HPLC elution, the semipreparative fraction was collected in 30mL of water. This solution was loaded on a Sep-Pak tC18 cartridge (pretreated with 4mL ethanol, followed by 20 mL water). After that, the cartridge was washed with 20 mL of water, followed by the elution of $[^{11}\text{C}]$JNJ-39594906 with 1 mL ethanol and either 4, 9 or 14 mL of PBS. The corresponding formulated solution of $[^{11}\text{C}]$JNJ-39594906 was measured for radioactivity and was analyzed on analytical HPLC to determine UV/radioactive purity, and specific activity. A 10 mL solution of $[^{11}\text{C}]$JNJ-39594906 was obtained with a radioactivity of 3,3 GBq, a radio chemical yield (RCY) of 10,5% and a specific activity (SA) of 238 GBq/µMol.
4.5 Autoradiography I
In preparation, 4 plates of adjacent object plates containing snap frozen, non-fixed brain slices of healthy male Wistar rats (cortex, hippocampus and thalamus) were taken from a -80 °C freezer. The slices were thawed to room temperature, and 4 times washed in Tris-HCl buffer (pH 7.4) for 5 minutes. Subsequently, the slices were dried and placed in incubation trays.

5 mL of a formulated [11C]JNJ-39594906 in 20% ethanol and 80% PBS were obtained. Quality control (QC) of the formulation showed a specific activity of 82 GBq/µmol, a precursor content of 1600 nM and a concentration of JNJ-39594906 of 800 nM. Following estimation by means of table 11 (see appendix 5), 11 µL of the formulated radioligand were diluted to 5 mL to obtain an incubation solution containing two times the \( K_i \) of JNJ-39594906 on human H\(_4\)R (7 nM), which was believed to be sufficient for the unknown rat \( K_i \).

Four different incubation solutions where prepared as shown in table 2: for the determination of the total binding of [11C]JNJ-39594906, and radioligand binding while blocking with thioperamide, blocking with JNJ-7777120 and blocking with JNJ-39594906.

Table 2: 4 different incubation solutions used in autoradiography.

<table>
<thead>
<tr>
<th></th>
<th>11 µL [11C]JNJ-39594906 formulation</th>
<th>+75 µL 1 mM Thioperamide solution</th>
<th>+25 µL 1 mM JNJ-7777120 solution</th>
<th>+15 µL 1 mM JNJ-39594906 solution</th>
<th>Fill to 5 mL with Tris-HCl (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total binding</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioperamide</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>JNJ-7777120</td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JNJ-39594906</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

The object plates were incubated with 1 mL of each solution for 30 minutes. After incubation, the slices were washed 3 times for 1 minute in ice-cold Tris-HCl buffer (pH 7.4) and dropped in ice-cold water for a short time. The slices were dried under an air flow. When dried, the plates were exposed to a phosphorimaging plate for 30 seconds, 3 minutes, 30 minutes and overnight. Between each exposure, the phosphorimaging plate was scanned in the phosphorimager, and erased with light. The scans had a resolution of 25 µm per pixel and a latitude of 4.

4.6 Autoradiography II
Again 4 adjacent object plates with brain parts of interest (cortex, hippocampus and thalamus) of healthy male Wistar rats were prepared for incubation (see chapter 4.5). Further, 4 adjacent object plates with LPS injected, snap frozen, non-fixed rat brain slices where taken out of the -80 °C freezer. Also these were thawed to room temperature and washed in Tris-HCl buffer (pH 7.4), 4 times for 5 minutes. All washed plates were dried and placed on the incubation trays.
5 mL of formulated $[^{11}C]$JNJ-39594906 in a 20/80% ethanol/PBS solution was obtained from a full radioligand preparation. The specific activity was determined to be 51 GB/µmol. The concentration of JNJ-39594906 was 7700 nM and precursor concentration was 1600 nM. Using table 11 (see appendix 5), a dilution of 9 µL radioligand formulation to 5 mL incubation solution to obtain a concentration of 2 times the $K_i$ of JNJ-39594906 on human $H_4R$ (7 nM).

The corresponding solutions for blocked and total binding were prepared as mentioned in the previous paragraph (table 2). The object plates were incubated for 30 minutes with 1 mL of each solution, one per healthy brain slice and one per LPS brain slice. Subsequently the plates were 3 times washed with ice-cold Tris-HCl for 1 minute, followed by a dip in ice-cold water. The radioactive plates were exposed to a phosphorimaging plate for 45 minutes and scanned with a resolution of 25 µm per pixel and a latitude of 4.

4.7 Biodistribution
The biodistribution study was split into two sessions of 8 rats, with a total of 16 healthy male Wistar rats. The biodistribution was determined for 5, 15, 30 and 60 with 4 rats at each time point. For the first series, 3.3 GBq of radioactive formulated $[^{11}C]$JNJ-39594906 were obtained with a SA of 110 GBq/µMol in a 10 mL 10/90% ethanol/PBS solution. For the second series again 3.3 GBq of $[^{11}C]$JNJ-39594906 formulation were produced with a SA of 73 GBq/µMol. All rats were injected with 195 ± 7 mg of formulation and 60 to 70 MBq of $[^{11}C]$JNJ-39594906, and sacrificed at the appointed time points. Blood and urine were collected. Subsequently the rats were dissected into heart, lungs, liver, kidney, tail, bone. The brain area was dissected in olfactory bulb, prefrontal cortex, cerebellum, hippocampus, hypothalamic region, cerebral cortex, brain stem and striatum. The organs were placed in preweighed counting tubes and counted for radioactivity. These tubes were weighed again to determine the mass of the organs. All tissue and blood values were expressed as percentage injected dose per gram tissue (%ID/g) and urine as percentage injected dose (%ID).

4.8 Metabolite analysis
To establish a suitable procedure for the metabolite analysis of $[^{11}C]$JNJ-39594906, two experiment series were conducted. First a matrix test with radioligand incubated serum was undertaken to determine the extraction efficiency and a suitable HPLC method. When the methods were confirmed the ex vivo rat metabolite analysis was performed.

4.8.1 Extraction method determination
To simulate the binding of $[^{11}C]$JNJ-39594906 deriving radioactive species on biological materials, 20 mL of stored inactive human serum were mixed with a low amount of formulated $[^{11}C]$JNJ-39594906 solution with a vortexer. Using this incubation mixture, five different methods were tested for their extraction capability.

For the development of a separation of the biological content and radioactive compounds via SPE, three different cartridges containing reversed phase stationary phases were tested: Sep-pak tC-18, Sep-pak tC-2 and conventional Sep-Pak C-18. On an SPE vacuum manifold, 1 mL of the incubated serum was loaded on the corresponding cartridge and the passed residue collected the so called polar fraction. Followed
by a washing step using 3 mL of H$_2$O or 3 mL of 6 mM HCl in H$_2$O, 3 mL of MeOH were used to extract the non-polar fraction of remaining radioactive species. All fractions and the cartridges were measured for radioactivity in a gamma counter.

4.8.2 Ex vivo metabolite analysis
Six healthy male wistar rats were injected with around 100 MBq (at the start of the experiment) of formulated $[^{11}C]$JNJ-39594906 into their tail vein while under isoflurane anesthesia. After 15 and 45 minutes p.i. the rats were sacrificed (3 rats per time point). Blood samples were obtained and the brain was removed. 4 mL of 40% acetonitrile in water was added to the brain. The brain was centrifuged for 4000 rpm for 5 minutes at 20 °C. The supernatant separated from the pallets and were counted for radioactivity in the Wallac Wizard automated gamma counter. After counting the supernatant was injected directly on the HPLC method for metabolite analyses (see chapter 4.1). The blood samples were collected in herapin tubes and centrifuged at 4000 rpm for 5 minutes at 4 °C. The tC-2 Sep-Pak columns were pretreated with 3 mL MeOH followed by 6 mL H$_2$O. 1 mL blood supernatant together with 2 mL was loaded on the Sep-Pak under vacuum. The column was washed with 3 mL of water (non-polar fraction), and eluted with 1,5 MeOH and 1,5 H$_2$O (polar fraction). All fractions and the cartridge were counted for radioactivity in the Wallac Wizard automated gamma counter. After that, 1 mL of the elution was injected on the HPLC method for metabolite analyses (see chapter 4.1).
5. Results and discussion
In the present thesis, new results are given for the precursor synthesis, radiosyntheses, purification, and the outcome of in vitro autoradiography studies, ex vivo biodistribution studies and metabolite analysis reported. The results of previous organic syntheses and optimizing of the radiosyntheses can be found in the preceding final report (van der Aa 2014).

5.1 Preparations of the precursor for radiosynthesis
The precursor compound is the desmethyl JNJ39758979 analogue of JNJ39758979. This compound is used for the reaction with $[^{11}\text{C}]\text{MeI}$ or $[^{11}\text{C}]\text{MeOTf}$ to result in the radioactive $[^{11}\text{C}]\text{JNJ39758979}$.

One synthesis for the preparation of precursor 4 was carried out based on (Cai, et al. 2013), with improvements described in (van der Aa 2014). This synthesis resulted in a high yield of 95% with 329 mg of the hydrochloride of 6 as a yellow solid. The mass was the same as calculated $[\text{M+H}]^+$ values and the mass described in the patent. The $^1\text{H}$-NMR spectrum was the same as found in the patent. No $^{13}\text{C}$-NMR data was collected in the patent, but with attached proton test (APT) every carbon atom was found in the obtained spectra.
5.2 Test reactions to optimize the radiosynthesis

Multiple test reactions were already performed for the development of the radiosynthesis of $[^{11}\text{C}]$JNJ-39594906 (van der Aa 2014). Nevertheless, in this report an improved method was used for the determination of the yield. Previously, samples were taken from the reaction diluted reaction mixture by a syringe, and analyzed with radio-HPLC. However, these mixtures contained volatile radioactive compounds, such as non-reacted $[^{11}\text{C}]$MeI, which could evaporate out of sample and “increase” the putative percentage of $[^{11}\text{C}]$JNJ-39594906 of the total radioactivity.

Therefore a new method was developed to determine the conversion of $[^{11}\text{C}]$MeI to $[^{11}\text{C}]$JNJ-39594906. After a certain reaction time, a stream of helium would bubble through the reaction mixture to evaporate all volatile compounds. The dispersed volatile compounds were trapped onto a Porapak cartridge. Before and after this helium stream, the radioactivity was measured of the reaction mixture and the Porapak and a sample from the volatile free reaction mixture injected in analytical HPLC. Thereby, a radioactivity balance could be obtained for every following radiosynthesis.

In table 3 an example for the determination of the radioactivity balance of a radiosynthesis is shown. The first column shows a time point followed by the related step. The third column shows what vial or cartridge is measured in the dosiscalibrator, followed by the corresponding radioactivity measured in MBq. The fifth column shows the activity corrected for radioactive decay. In this way it is possible to determine the overall percentages, shown in column eight and nine. The first displays percentages from the starting activity obtained in reaction vial I (RV-I). The second displays the percentage of radioactivity from start radioactivity measured in reaction vial II (RV-II).

Table 3: An example of a radioactivity balance.

<table>
<thead>
<tr>
<th>03-04-14</th>
<th>Approach: 0,5 mg precursor / 0,5 ml DMSO / triflate / 5min 120 °C</th>
<th>$N_t = N_0 \times e^{(-t\ln2)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Act [MBq]</td>
<td>Timefile [h]</td>
</tr>
<tr>
<td>14:24</td>
<td>Act trapped in RV-I</td>
<td>9100</td>
</tr>
<tr>
<td>14:30</td>
<td>Act trapped in RV-II before reaction</td>
<td>1300</td>
</tr>
<tr>
<td>14:32</td>
<td>Act trapped in Porapak before reaction</td>
<td>130</td>
</tr>
<tr>
<td>14:36</td>
<td>Act in RV-II after reaction</td>
<td>1031</td>
</tr>
<tr>
<td>14:46</td>
<td>Act in RV-II after helium flow (Product)</td>
<td>470</td>
</tr>
<tr>
<td>14:47</td>
<td>Act in Porapak after helium flow</td>
<td>229</td>
</tr>
</tbody>
</table>

Analytical HPLC Conversion: $[^{11}\text{C}]$JN39594906 % 42,00

For instance, the raw product in table 3 was measured to contain 470 MBq. Corrected for decay, the synthesis resulted then in 995 MBq of radiolabelled species. This corresponded to 10% of the starting activity trapped in RV-I, and to 62% of the activity trapped in RV-II. However, in this case analysis with HPLC revealed only 45% of the radioactivity in the sample corresponding to $[^{11}\text{C}]$JNJ-39594906. This resulted in 5% overall radiochemical yield and only 28% conversion of $[^{11}\text{C}]$MeI to the radioligand. Table 4 shows all results for the radiochemical outcome determined by use of this method.
This table also renders the course of the experimental determination of the best possible conditions for a radiosynthesis. The main goal was to increase the conversion of $[^{11}C]$MeI to the radioligand in RV-II. Whereas the trapping of $[^{11}C]$CO$_2$ and its conversion to $[^{11}C]$MeI of usually around 33% offers not too much room for improvements. The nucleophilic substitution of the primary amine 4 with $[^{11}C]$MeI to obtain $[^{11}C]$JNJ-39594906 is a novel approach and can be optimized.

The first and main improvement step was achieved by using precursor 4 in form of its free base instead of an ammonium hydrochloride salt. This increased the conversion of $[^{11}C]$MeI from 11% to 38%. For this purpose, a stock solution was created of 1mg/mL precursor 4 in DMSO. By this, the amount of precursor was kept constant for all radiosyntheses. Furthermore, potential decomposition was investigated and precursor 4 also as free amine found to be stable for at least 3 months, if kept in the freezer at –8 °C.

The determination of DIPEA as best suitable base was already discovered in previous investigations (van der Aa 2014). The base was furthermore added about 12 hours before the radiosynthesis to the precursor 4 as a free base in solution. This increased the radiochemical yield to about 15% (entry 6, table 4), and was performed for every radiosynthesis after this discovery.

Apart from that, the use of $[^{11}C]$methyltriflate ($[^{11}C]$MeOTf) as radiolabelling agent was investigated under different conditions. Reacting at 20 °C resulted in almost no yield, and at 70 °C and without a base, a yield of only 22% was achieved, comparable to the results obtained with methyliodide before. For the method using $[^{11}C]$MeI at 70 °C for 5 minutes using DIPEA, the conversion was 33% ±11% (n=4). When $[^{11}C]$MeOTf was used in combination with DIPEA, conversions of 28-33% were achieved. The

<table>
<thead>
<tr>
<th>Solvent / Temperature / Time / Methylation agent</th>
<th>Precursor [mg]</th>
<th>Base</th>
<th>$[^{11}C]$JNJ39594906 Yield from RV-I</th>
<th>Conversion RV-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 salt</td>
<td>5µL NaOH</td>
<td>69,03</td>
<td>2,02 (split)</td>
</tr>
<tr>
<td>300 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 salt</td>
<td>5µL DIPEA</td>
<td>77,23</td>
<td>2 (split)</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>No base</td>
<td>77,23</td>
<td>7,55</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>No base</td>
<td>69,97</td>
<td>10,85</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>10µL NaOH</td>
<td>1,31</td>
<td>0.3 (split)</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>10µL DIPEA</td>
<td>77,53</td>
<td>11.85 (split)</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>10µL DIPEA</td>
<td>68,56</td>
<td>7,55</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>No base</td>
<td>70,01</td>
<td>10,11</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>10µL DIPEA</td>
<td>77,72</td>
<td>3,79</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>10µL DIPEA</td>
<td>52,07</td>
<td>3,79</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>No base</td>
<td>52,02</td>
<td>7.94 (split)</td>
</tr>
<tr>
<td>500 µL DMSO, 70 °C, 5 min, Mel</td>
<td>0.5 freebase</td>
<td>No base</td>
<td>44,76</td>
<td>5.42 (split)</td>
</tr>
</tbody>
</table>

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The results of both approaches are in the same range, therefore the following full radiosyntheses, described in chapter 5.3 were conducted under both conditions.

### 5.3 Complete radiosyntheses of \([^{11}C]\)JNJ-39594906

The full syntheses of \([^{11}C]\)JNJ-39594906 including purification and formulation were performed with the best conditions found while radiosynthesis optimizations (chapter 5.2.). Following from a total radiosynthesis, the overall decay corrected radiochemical yield (RCY) of isolated \([^{11}C]\)JNJ-39594906 at the end of synthesis (EOS) can be determined. Apart from radiochemical yields calculated based on the starting activity and the conversion from \([^{11}C]\)MeI in RV-II, the specific activity of the radiotracer is determined, which quantifies the amount of radioactive compound per unit weight or molecular amount of overall compound present in the sample. All full radiosyntheses carried out are listed in table 5.

#### Table 5: Methods and results of all complete radiosynthesis of \([^{11}C]\)JNJ-39594906.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>10 DIPEA</td>
<td>5</td>
<td>70</td>
<td>Mel</td>
<td>Method A</td>
<td>No</td>
<td>9,25</td>
<td>16,47</td>
<td>4,23</td>
<td>22776</td>
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<td>2</td>
<td>10 DIPEA</td>
<td>5</td>
<td>70</td>
<td>Mel</td>
<td>Method A</td>
<td>No</td>
<td>9,86</td>
<td>18,48</td>
<td>6,14</td>
<td>626</td>
<td>0,20</td>
</tr>
<tr>
<td>3</td>
<td>10 DIPEA</td>
<td>5</td>
<td>70</td>
<td>Mel</td>
<td>Method A</td>
<td>No</td>
<td>5,21</td>
<td>12,9</td>
<td>15,76</td>
<td>11211</td>
<td>1,70</td>
</tr>
<tr>
<td>4</td>
<td>10 DIPEA</td>
<td>5</td>
<td>70</td>
<td>MeOTf</td>
<td>Method A</td>
<td>No</td>
<td>8,59</td>
<td>21,54</td>
<td>2,86</td>
<td>6746</td>
<td>0,30</td>
</tr>
<tr>
<td>5</td>
<td>10 DIPEA</td>
<td>5</td>
<td>100</td>
<td>MeOTf</td>
<td>Method A</td>
<td>No</td>
<td>9,15</td>
<td>24,81</td>
<td>2,01</td>
<td>6050</td>
<td>0,40</td>
</tr>
<tr>
<td>6</td>
<td>10 DIPEA</td>
<td>5</td>
<td>120</td>
<td>MeOTf</td>
<td>Method A</td>
<td>No</td>
<td>11,86</td>
<td>28,67</td>
<td>5,94</td>
<td>42241</td>
<td>2,00</td>
</tr>
<tr>
<td>7</td>
<td>10 DIPEA</td>
<td>5</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method C</td>
<td>8,26</td>
<td>19,99</td>
<td>8,35</td>
<td>448</td>
<td>0,30</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>5</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method C</td>
<td>8,52</td>
<td>21,75</td>
<td>12,34</td>
<td>369</td>
<td>0,30</td>
</tr>
<tr>
<td>9</td>
<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method D</td>
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<td>20,52</td>
<td>107,32</td>
<td>2138</td>
<td>3,00</td>
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<td>10</td>
<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method D</td>
<td>8,44</td>
<td>22,43</td>
<td>80,30</td>
<td>1360</td>
<td>2,90</td>
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<tr>
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<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method D</td>
<td>10,18</td>
<td>21,67</td>
<td>81,78</td>
<td>829</td>
<td>2,80</td>
</tr>
<tr>
<td>12</td>
<td>10 DIPEA</td>
<td>5</td>
<td>70</td>
<td>Mel</td>
<td>Method B</td>
<td>Method D</td>
<td>6,51</td>
<td>11,86</td>
<td>51,48</td>
<td>1600</td>
<td>1,90</td>
</tr>
<tr>
<td>13</td>
<td>10 DIPEA</td>
<td>5</td>
<td>70</td>
<td>Mel</td>
<td>Method B</td>
<td>Method E</td>
<td>6,20</td>
<td>10,24</td>
<td>54,39</td>
<td>544</td>
<td>1,70</td>
</tr>
<tr>
<td>14</td>
<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method E</td>
<td>8,28</td>
<td>20,61</td>
<td>75,57</td>
<td>797</td>
<td>1,90</td>
</tr>
<tr>
<td>15</td>
<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method E</td>
<td>8,79</td>
<td>23,89</td>
<td>65,13</td>
<td>269</td>
<td>2,35</td>
</tr>
<tr>
<td>16</td>
<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method E</td>
<td>11,65</td>
<td>25,69</td>
<td>109,55</td>
<td>308</td>
<td>3,25</td>
</tr>
<tr>
<td>17</td>
<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method E</td>
<td>10,41</td>
<td>25,06</td>
<td>238,81</td>
<td>889</td>
<td>3,33</td>
</tr>
<tr>
<td>18</td>
<td>10 DIPEA</td>
<td>3</td>
<td>120</td>
<td>MeOTf</td>
<td>Method B</td>
<td>Method E</td>
<td>11,85</td>
<td>25,94</td>
<td>73,40</td>
<td>519</td>
<td>3,32</td>
</tr>
</tbody>
</table>

Total radiosyntheses of \([^{11}C]\)JNJ-39594906 were carried out with low starting radioactivity amounts (10 ± 1 GBq, displayed in white rows) for solely synthesis validation and with higher starting activities (77 ± 15 GBq, light green rows) to produce a radiotracer for the preclinical investigations. The average trapping of methyl iodide in RV-II was 53 ± 8% of the total radioactivity, whereas for methyl triflate the average trapping was 40 ± 4%. At first methyl iodide was used as methylation agent, however in the previous paragraph was found that methyl triflate had less variance in yields. That is the reason methyl triflate is used more for full synthesis.
In the first instance, methyliodide was used as methylation agent since the trapping is high, it usually methylates free amines and alcohols sufficiently and it takes less preparation time compared to methyltriflate. However in the course of radiosynthesis optimization (chapter 5.2.) it was found that the use of methyltriflate did not cause higher conversion but had less variance in the radiochemical yields. This higher reliability was the reason for the application of methyltriflate in most of the full radiosyntheses. Overall could be observed that the conversion of methyltriflate in the total radiosyntheses was higher than in radiolabelings with methyliodide. The radiochemical yields are somewhat higher for methyltriflate, increased by 3.6%. Methylation with methyltriflate was used for further syntheses.

Table 6: Yields and conversions of MeI versus MeOTf compared

<table>
<thead>
<tr>
<th></th>
<th>Average</th>
<th>± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall yield MeI</td>
<td>6,0</td>
<td>0,7</td>
</tr>
<tr>
<td>(Entry nr. 3, 12 and 13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conversion MeI</td>
<td>11,7</td>
<td>1,3</td>
</tr>
<tr>
<td>(Entry nr. 3, 12 and 13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall yield MeOTf</td>
<td>9,6</td>
<td>1,7</td>
</tr>
<tr>
<td>(Entry nr. 9-11 and 14-18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conversion MeOTf</td>
<td>23,2</td>
<td>0,5</td>
</tr>
<tr>
<td>(Entry nr. 9-11 and 14-18)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To ascertain the purity and specific activity of \(^{11}\text{C}\)JNJ-39594906 in the final preparations by use of analytical HPLC, two calibration curves were created based on different concentrations of JNJ-39594906 and precursor 4, see appendix 4. The latter turned out to be an issue as small portions of precursor eluted together with the product creating an impurity.

Based on the calibration curve first the concentration of JNJ-39594906 in the formulation was determined. The specific activity at EOS was determined then dividing the radioactivity of \(^{11}\text{C}\)JNJ-39594906 by the concentration of overall compound in µMol. The concentration of the (radio)ligand had to especially be known for the incubation of brain slices in autoradiography studies. In these studies an optimum amount of radiotracer for a high receptor occupancy, but low saturation of non-specific binding sites should be applied. Further, the specific activity is always wanted to be as high as possible, so that the formulation solution contains as low non-radioactive JNJ-39594906 as possible. High amounts of non-radioactive JNJ-39594906 would occupy H₄R positions as well and make them unavailable for \(^{11}\text{C}\)JNJ-39594906 binding and therewith detection.

The specific activity of \(^{11}\text{C}\)JNJ-39594906 was increased when high radioactivity beams were used, because the radioactivity is 5-7 times as high as with a low radioactivity beam, which increases the amount of radioactive \(^{11}\text{C}\)CO₂ compared to the omnipresent non-radioactive one. To avoid non-radioactive CO₂, the pipelines from the cyclotron to the liquid nitrogen trap are flushed before every transfer of \(^{11}\text{C}\)CO₂. Furthermore the flushing of the lines from the nitrogen trap to the synthesis unit is important, and was done insufficiently until radiosynthesis no. 8. Thereafter and with more cautious preparation a big increase in specific activity was achieved.

For the purification and preparation of \(^{11}\text{C}\)JNJ-39594906, first the semipreparative HPLC method A was used (85:15:0.2 H₂O:EtOH:DIPA, 3mL/min) and the formulation occurred without any further solid phase extraction only by dilution of the collected product fractions. This method was used as timesaving and as
it was applicable at a partner institutes facilities (Department of Radiopharmacy, KU Leuven). However, using ethanol as an organic eluent instead of acetonitrile caused tailing of precursor 4 as well as the radioligand. This made the separation of radioligand and precursor and the efficient collection of product fraction difficult. When determining the purity of the [\(^{11}\)C]JNJ-39594906 formulation, comparably high amounts of precursor were observed with an average of 15 ± 15 µMol/L.

Because of that, method B was developed (85:15:0.2 H\(_2\)O:MeCN:DIPA, 3mL/min), followed by a formulation by solid phase extraction (SPE). The retention time decreased from 20 minutes to 12 minutes, by changing the organic eluent to acetonitrile. Also significantly less precursor 4 was observed in the final formulated mixture, due less tailing of precursor on the C-18 column. Furthermore, solid phase extraction could improve this separation because of the higher polarity of the precursor than of [\(^{11}\)C]JNJ-39594906 and therewith less absorption on a C-18 phase.

Three different methods for formulation via SPE where investigated. Thereby the initial loading of the column was the same for every method. Product fractions collected from semi preparative HPLC were diluted in 30 mL of water and loaded on the Sep-Pak tC18 cartridge, followed by a washing step with 20 mL of water. Also no radioactivity was lost in the waste. In SPE Method C 1 mL of ethanol was then used to elute the radioligand, followed by 14 mL of PBS, resulting in a 6.6% ethanol/PBS formulation. In Method D 1 mL of ethanol was used and 4 mL PBS to obtain a 20% ethanol/PBS formulation. Finally, method E was applied to obtain a 10% ethanol/PBS formulation by using 1 mL of ethanol and 9 mL of PBS for elution.

As mentioned above, the amount of precursor in the formulation decreased a lot by using acetonitrile as organic eluent. For method C, the precursor amount was 369-448 nMol/L (n=2), for method D 1482 ± 543 nMol/L (n=4) and with method E the radioligand formulation contained 554 ± 251 nMol/L (n=6) of precursor 4. Changes in the formulation methods have been only made to increase the concentration of radioligand and decrease the content of ethanol. Variation of PBS amounts did not alter the elution of the radioligand. Method D is not suitable for \textit{in vivo} studies, because the concentration of ethanol is too high to be injected in animals. Method E is not too much diluted, but still useable for autoradiography.

By changing the preparation of [\(^{11}\)C]JNJ-39594906 from semi preparative HPLC with an ethanol containing eluents and direct formulation to an acetonitrile containing eluent and formulation via SPE, the overall radiosynthesis time did not increase. When ethanol was used, the total synthesis time was 33 ± 3 minutes (n=6). By using acetonitrile and an SPE formulation method the total synthesis time averaged 28 ± 2 minutes (n=12).
5.4 Autoradiography I

At first, it was to determine how long the phosphor imaging screens had to be exposed to radioactive material in relation to the applied radioactivity of incubation solutions. Furthermore, it had to be assessed whether the H₄R is available for imaging in the healthy rat brain with [¹¹C]JNJ-39594906.

Four different exposure times were tested: 30 seconds, followed by 3 minutes, 30 minutes and an overnight exposure. Both 3 minutes and 30 minutes of exposure resulted in clear images without much background. The 30 seconds exposure was too short and decreased the tissue to background ratio. The same was found for the overnight exposure, since the main radioactivity already decayed, and most of the excitation was deriving from the environmental background.

The results of the 30 min exposure of phosphors imaging screen development are depicted in figure 12. The corresponding numerical values of both optimal exposure times can be found in table 7. These numbers were generated by outlining the brain with the quantification software. The program can determine the average intensity per pixel. The values from 30 minutes exposure are on average 5.4 times as higher as the 3 minutes exposure.

First of all, no specific brain region could be identified with increased or decreased radioligand binding. Furthermore, visual as well as quantification results of this autoradiography study are not as expected. The slides for determination of total radioligand binding (No. 1, figure 12), means no competition with other H₄R inhibitors, do not show the most intensive blackening and highest values for average intensity. Instead, the thioperamide blocking revealed highest values for average intensity and the darkest images. Although thioperamide is reported as an antagonist for the H₃R and H₄R with an $K_i$ value of 28 nM for rat H₄R, the coincubation with a concentration of 500 times that $K_i$ value could not alter the binding of [¹¹C]JNJ-39594906. Even if the binding of [¹¹C]JNJ-39594906 to brain matter is only of non-specific nature and therewith not inhibited by Thioperamide, then the radioligand binding under blockade should be at least the same as for the non-blocked condition.

At least the JNJ-7777120 blocking slides (No. 3, figure 12) showed as expected lower values for the average intensity and decreased radioligand binding on the images, as this solution contained the highest concentration of the competing antagonist with <1200 times the $K_i$ on rat H₄R of 4 nM. This indicates an efficient blockade of specific radioligand binding on H₄R binding positions.
The same observations as for the blocking experiment with Thioperamide were made under homologous competition with JNJ-39594906 (No. 4, figure 12). Those brain slides also show higher radioligand binding than the total binding slides. Unfortunately, in the course of the preclinical study, the affinity of non-methylated analogue JNJ-39758979 on rat H4R was published to be only about $K_i = 188$ nM, with a high standard deviation of 66 nM. Nevertheless, as the brain slices were coincubated with about 400 times the $K_i$ of JNJ-39594906 on human, homologous competition should at the most show no blockade, hinting to a high non-specific binding of $[^{11}C]$JNJ-39594906.

Table 7: Numerical values counted by the phosphorimager.

<table>
<thead>
<tr>
<th>Method</th>
<th>Average Intensity 30 minutes exposure [counts]</th>
<th>Average Intensity 3 minutes exposure [counts]</th>
<th>Percentage of counts from Total binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Total binding</td>
<td>19130</td>
<td>3670</td>
<td>112%</td>
</tr>
<tr>
<td></td>
<td>17377</td>
<td>3377</td>
<td>110%</td>
</tr>
<tr>
<td>2 Thioperamide blocking</td>
<td>20383</td>
<td>3889</td>
<td>118%</td>
</tr>
<tr>
<td></td>
<td>21468</td>
<td>4007</td>
<td>114%</td>
</tr>
<tr>
<td>3 JNJ7777120 blocking</td>
<td>16909</td>
<td>3191</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>15803</td>
<td>2938</td>
<td>91%</td>
</tr>
<tr>
<td>4 JNJ39594906 blocking</td>
<td>20995</td>
<td>3707</td>
<td>115%</td>
</tr>
<tr>
<td></td>
<td>21252</td>
<td>3766</td>
<td>116%</td>
</tr>
</tbody>
</table>

Both results of blocking with Thioperamide and JNJ-3959406 are unexpected. In vitro, there is no explanation for higher radioligand binding while a competing substance is present in the same incubation solution. An experimental failure while preparing the incubation solutions was assumed to be responsible for the strange outcome. Therefore, a new autoradiography study was performed to confirm or disapprove the results.
5.5 Autoradiography II

The second autoradiography study was done on 4 slides of healthy male Wistar rat brains and 4 rat brain slices with induced neuroinflammation by stereotactic LPS injection. New incubation solutions were prepared containing the same concentrations of \(^{11}\text{C} \)JNJ-39594906 and inhibitors as in the previous autoradiography study.

Following the same incubation and washing protocol as above, the radioactive brain slides were exposed about 45 min to the phosphor imaging screens. The numerical values deriving from scanning and processing with the imager software can be found in table 8.

The second autoradiography study revealed comparably unrespectable results. The total binding experiment showed the lowest average intensity of all incubated brain slides. For the blocking with JNJ-39594906 (No. 4, figure 13) at 400 times the \( K_i \) on human \( H_4 \)R again an increase of the total intensity was observed, which is unexpected since the radioactive and non-radioactive compound definitely compete for the exact same binding sites. Unlike the previous autoradiography study, no blocking occurred with JNJ-7777120. Again, coincubations with Thioperamide and JNJ-39594906 showed an increased radioligand binding as in the previous experiments.

Table 8: Numerical values for the second autoradiography study.

<table>
<thead>
<tr>
<th>Method</th>
<th>Average Intensity 45 minutes exposure [counts]</th>
<th>Percentage of counts from Total binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Total binding</td>
<td>4599</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5397</td>
<td></td>
</tr>
<tr>
<td>2 Thioperamide blocking</td>
<td>5756</td>
<td>115%</td>
</tr>
<tr>
<td></td>
<td>5462</td>
<td>109%</td>
</tr>
<tr>
<td>3 JNJ7777120 blocking</td>
<td>5451</td>
<td>109%</td>
</tr>
<tr>
<td></td>
<td>5652</td>
<td>113%</td>
</tr>
<tr>
<td>4 JNJ39594906 blocking</td>
<td>6314</td>
<td>126%</td>
</tr>
<tr>
<td></td>
<td>7423</td>
<td>149%</td>
</tr>
</tbody>
</table>

Apart from the same behavior of the overall radioligand binding as for the healthy brains, no conclusions can be made regarding the neuroinflammatory foci from the LPS slices (left side, figure 13). The white center of the LPS model brain slides images demonstrate removed and damaged brain material. In case of increased \( H_4 \)R activity while neuroinflammation, LPS injected brains are expected to show two
different sites of radioligand binding intensity because of the stereotactic LPS injection in one of the brain halves. This could not be observed from this decomposing rat brain material and will be investigated again on freshly prepared slices of this neuroinflammation model.

A high amount of radioactivity can be found on the glass part of the slides, and is not sufficiently washed off. This could be a hint for the incubation solution to be too sticky, resulting in not only sticking to the glass, but also on the brain itself. That’s why alternate incubation buffers were tested. However no significant decrease was found using either citric acid buffer, acetic acid buffer or 2,5% tween 80 in 9mM NaCl as incubation solution.

The precursor compound free amine 4 or JNJ-39758979 was found to have a $K_i$ value on rat H$_4$R of 188 ± 61. This article also published the rat $K_i$ to be 5,4 ± 0,3. These values could be an explanation for the insufficient blocking of the H$_4$R, however, the rat and mouse H$_4$R are very similar, and such differences in values are not expected. Also the standard error of the mean is very high which makes the result even less likely.

Further autoradiography studies will be performed to clarify the obtained results. Changes in blocking substances could be an option for improvement. Also increasing the blocking from 500 x $K_i$ to 1000x $K_i$. However this would be unlikely to have an impact on the results, since blocking should be sufficient either way.
5.6 Biodistribution

At all investigated time points, high values of radioactivity were found in the periphery of the rats (figure 14). Fast clearance of $[^{11}C]$JNJ-39594906 and its possible metabolites was observed via the urinary pathway with rapid uptake and following washout from the kidneys and with high concentrations of radioactivity in the urine ($\pm$30% of the injected dose at 15 and 30 min p.i.). Notably, very low concentrations of radiotracer are observed in the blood with 0.13 %ID/g at 5 min and 0.6 %ID/g at 15 min p.i., and remaining constant with around 0.10 %ID/gram over all investigated time points.

Figure 14: Biodistribution displayed per organs in %ID/gram.
Also in all investigated brain regions, a very low tracer uptake of 0.07 %ID/g was observed on average. This low radioactivity concentration could be even based on the remaining blood in the brain tissue, as for all brain regions this value is remaining constant over all investigated time points. This is indicating that the tracer does not enter the brain via the BBB. Another explanation for such a low cerebral radiotracer concentration might be fast metabolism (see chapter 5.7.2) and the high and very probably non-specific binding in the periphery. Both cause that there is not enough radiotracer remaining in the blood and therewith also not available for an uptake in the brain. Less probably and if a very fast uptake in the brain was taking place (before the investigated 5 min p.i.), a very low expression of H₄R in healthy rat brain and therewith not enough binding positions for [¹¹C]JNJ-39594906 could cause a very fast washout from the brain. It is known that H₄R is present in the healthy rat brain (Strakhova, et al. 2009), but the concentrations of active receptor are not known so far.
5.7 Metabolite analysis

5.7.1 Extraction method determination
For their extraction capacity, five different extraction methods varying the stationary phase of the cartridge, the washing step and the eluent were tested one to two times. The mean values from this experiment can be found in table 9.

Table 9: Extraction values found in test extractions for [11C]JNJ-39594906 out of serum.

<table>
<thead>
<tr>
<th></th>
<th>Sep-Pak tC-18 with H2O (n=1)</th>
<th>Sep-Pak tC-18 with 6 mM HCl (n=2)</th>
<th>Sep-Pak tC-2 with H2O (n=2)</th>
<th>Sep-Pak tC-2 with 6 mM HCl (n=2)</th>
<th>Sep-Pak C-18 with H2O (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma %</td>
<td>0.3</td>
<td>6.6 – 8.9</td>
<td>0.2 – 0.2</td>
<td>36.7 – 32.9</td>
<td>0.3 – 0.2</td>
</tr>
<tr>
<td>H2O %</td>
<td>0.0</td>
<td>10.8 – 10.5</td>
<td>0.0 – 0.0</td>
<td>56.9 – 56.7</td>
<td>0.1 – 0.1</td>
</tr>
<tr>
<td>MeOH %</td>
<td>85.3</td>
<td>81.2 – 79.0</td>
<td>90.6 – 90.4</td>
<td>5.3 – 10.3</td>
<td>66.0 – 63.0</td>
</tr>
<tr>
<td>Cartridge %</td>
<td>14.4</td>
<td>1.4 – 1.5</td>
<td>9.3 – 9.4</td>
<td>1.1 – 1.0</td>
<td>33.6 – 36.8</td>
</tr>
</tbody>
</table>

The most efficient extraction method for metabolite analyses turned out to be the use of a Sep-Pak tC-2 for separation, after loading with the biological material washing with H2O and elution with methanol. Polar fractions, means the plasma and H2O are free of radioligand, which means all parent compound can be extracted with the non-polar fraction (MeOH).

5.7.2 Ex vivo metabolite analysis
Generally a low content of radioactivity was observed in plasma at 15 as well as 45 min p.i. Nevertheless, at both investigated time points, the amount of intact [11C]JNJ-39594906 in plasma was determined to be about 34%, see table 10, indicating a moderate metabolism in the periphery. Two structurally unidentified radiometabolites were determined by HPLC analysis of the non-polar fraction, as shown in figure 15. Also the mean concentrations of those metabolites remain almost constant in the course of the experiment.

Table 10: Results of the metabolite analysis in rat plasma.

<table>
<thead>
<tr>
<th>% Radioactivity</th>
<th>Intact tracer</th>
<th>Non polar metabolite 1</th>
<th>Non polar metabolite 2</th>
<th>Activity left on cartridge (Non-polar)</th>
<th>Polar fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 minutes p.i.</td>
<td>34.6 ± 13.9</td>
<td>3.8 ± 1.6</td>
<td>26.2 ± 10.9</td>
<td>8.9 ± 1.0</td>
<td>29.9 ± 4.6</td>
</tr>
<tr>
<td>45 minutes p.i.</td>
<td>34.0 ± 22.5</td>
<td>8.6 ± 2.9</td>
<td>22.6% ± 9.8</td>
<td>7.0 ± 1.4</td>
<td>39.7 ± 20.7</td>
</tr>
</tbody>
</table>
Figure 15: HPLC chromatograms deriving from collected and gamma counted fractions of the non-polar plasma fraction. The intact radiotracer has a retention time of 12 minutes.

Despite the in plasma still present parent $[^{11}\text{C}]$JNJ-39594906 and as already observed in the biodistribution studies (chapter 5.6), the uptake of radioactive compounds in the brain was very weak. Although the extracted content of radioactivity from the brain homogenate in the non-polar fraction was about 50% for 15 as well as 45 min p.i., it was impossible to detect any metabolite or intact $[^{11}\text{C}]$JNJ-39594906 using analytical HPLC. The content of radioactivity in the collected HPLC fractions was also too low to obtain a suitable chromatogram via gamma counting of those fractions.

Both together the results are also not satisfying regarding their reliability. Because of the weak concentrations of radioactivity in blood and brain, all investigated samples showed very low values for their gamma counts per minute, increasing the standard error of the measurements.

The high content of radioactivity in the polar fraction of the plasma can be correlated to the fast clearance of radioactivity via the kidney and urine, as found in the biodistribution studies. As $[^{11}\text{C}]$JNJ-39594906 was not rinsed through the cartridge with the water phase (chapter 5.7.1.), this observations hint to a fast metabolism to a very polar radiometabolite. Around 8% of radioactivity remaining on the cartridges after methanol elution could be based on an efficient binding of $[^{11}\text{C}]$JNJ-39594906 to lipophilic matrix. This goes along with the fact that high values of radioactivity were found in organs and tissues already at early time points in the biodistribution studies.

Combining the results of the biodistribution and metabolite analyses it can be concluded that $[^{11}\text{C}]$JNJ-39594906 is quickly cleared from the organism, and that for PET imaging insufficient amounts of intact radiotracer can be detected in the brain.
6. Conclusion

The novel radioligand $[^{11}C]$JNJ-39594906 was developed and investigated as potential radiotracer for the imaging and quantification of histamineH$_4$ receptor using PET.

$[^{11}C]$JNJ-39594906 was synthesized from its desmethyl analogue with a decay corrected radiochemical yield of 9.6 ± 1.7% using $[^{11}C]$MeOTf and yielding 6.0 ± 0.7% using $[^{11}C]$MeI. Starting from about 60 GBq of $[^{11}C]$CO$_2$, formulated $[^{11}C]$JNJ-39594906 could be produced in amounts of up to 3.3 GBq in 10 mL. This was sufficient for preclinical studies in vitro as well as in vivo.

$[^{11}C]$JNJ-39594906 was first used for in vitro autoradiography studies to determine the binding to the H$_4$R in the healthy rat brain. Unfortunately the non-specific binding was high, specific binding to the target could not be observed and could also not be blocked with other H$_4$R antagonists. These results in vitro hint to a concentration of H$_4$R in the healthy brain being too low for detection by PET and demonstrate the necessity to investigate this target in animal models with altered H$_4$R expression. In the ex vivo metabolite analyses, only 35% of intact $[^{11}C]$JNJ-39594906 were found in the blood after 15 minutes p.i.. High amounts of polar metabolites were observed and remained constant over the observed period too. These results correspond with the ex vivo biodistribution studies, were already after 15 minutes 30% of the injected dose were found in the urine, demonstrating a fast clearance of $[^{11}C]$JNJ-39594906 from the organism via polar metabolites. In brain, only 0.05% of the injected dose per gram brain tissue could be observed during the whole course of the biodistribution study, demonstrating no specific binding to a biological target and being too low for registration via PET. Following from that, neither $[^{11}C]$JNJ-39594906 nor radio metabolites could be identified in the HPLC analyses of brain extractions.

Altogether, the high non-specific binding in vitro, fast metabolism and insufficient brain uptake in vivo make $[^{11}C]$JNJ-39594906 not suitable for targeting H$_4$R in the rat brain.
7. Future perspective

\(^{11}\text{C}\)JNJ-39594906 was found to be an insufficient radiotracer to image H\(_4\)R in the rat brain by PET. This is found to be a setback in the search for a viable H\(_4\)R PET ligand and limits possibilities for the investigation of H\(_4\)R as suitable target for the imaging of neuroinflammation. The main focus for the next period will only be on the validation of \(^{11}\text{C}\)JNJ-7777120 in rats with induced neuroinflammation (Funke, Smits, et al. 2014).

It is currently still unknown if H\(_4\)R is an ideal receptor as target for the diagnosis and quantification of neuroinflammation. However a qualified radiotracer could confirm or disapprove this. Therefore, also the development and investigation of alternative H\(_4\)R ligands will be continued at the working groups of the radionuclide center and the department of medicinal chemistry at the VU Amsterdam.

Apart from the research for a new H\(_4\)R ligand and the H\(_4\)R as target for brain PET, multiple other receptors and enzymes are targeted for imaging and quantification of neuroinflammation, for instance the translocator protein (TSPO), purinergic receptors such as the P2X7, Monoamino-oxidase B (MAO-B), Matrix metalloproteinases (MMPs) and the cannabinoid receptor type 2 (CB2). The INMiND consortium will continue this search for appropriate PET-tracers for neuroinflammation.
8. Acknowledgements
The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° HEALTH-F2-2011-278850 (INMiND). I want to thank E. Kooijman, A. D. Windhorst, R. C. Schuit and M. Rongen for their help in ex vivo and in vitro animal studies. I want to thank Uta Funke for the hours she invested as my supervisor and her guidance in this project.
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I. Appendix 1: $^1$H-NMR of the radiosynthesis precursor

Figure 16: NMR spectrum of free amine precursor 4
II. Appendix 2: ATP $^{13}$C-NMR of the radiosynthesis precursor

Figure 17: $^{13}$C ATP NMR spectrum of free amine precursor 4
III. Appendix 3: Control scheme in-house synthesis unit

Figure 18: Control scheme of the in-house synthesis unit.
**IV. Appendix 4: Calibration curves of precursor and JNJ-39594906**

**Figure 19:** Calibration curve for the determination of JNJ-39594906 in a formulation

\[ y = 335522x - 38,665 \]

\[ R^2 = 0.9971 \]

**Figure 20:** Calibration curve for the determination of precursor in a formulation

\[ y = 355766x - 17,847 \]

\[ R^2 = 0.9994 \]
V. Appendix 5: Table for determination of the dilution needed for autoradiography.

<table>
<thead>
<tr>
<th>Concentration (μmol/ml)</th>
<th>Dilution needed for autoradiography</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1:20</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1:40</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1:200</td>
<td></td>
</tr>
</tbody>
</table>

Figure 21: Dilution graph for determination of the concentration of JNJ-39594905 in a formulation.