Synthesis, radiolabelling and preclinical evaluation of novel NMDA ligands as potential tracers for positron emission tomography

Pieter J. Klein

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Synthesis, radiolabelling and preclinical evaluation of novel NMDA ligands as potential tracers for positron emission tomography

door

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Chapter 1.

Introduction
1.1. NMDA receptor

Glutamate is the principal excitatory neurotransmitter, which plays an important role in neurotransmission via metabotropic and ionotropic receptors [1,2]. The N-methyl-D-aspartate receptor (NMDAr) complex is one of the members of the ionotropic glutamate receptor family. The other members are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAr) and the kainate receptor [3-6].

NMDArs are necessary for long term potentiation and they are thought to play an important role in learning and memory [7,8]. Deregulation of NMDArs is thought to be involved in several neurological and neuropsychiatric disorders, such as schizophrenia, mood disorders, Alzheimer’s disease, and Huntington’s disease [9].

The NMDAr (Figure 1) is a heterotetrameric assembly of four subunits [10,11]. To date, three types of subunits are known: the glycine-binding NR1 subunit with eight splice variants (NR1a-h), the glutamate-binding NR2 subunit encoded by four distinct genes (NR2A-D), and the glycine binding NR3 subunit encoded by two genes (NR3A-B) [12-17]. This assembly together forms a ligand-gated ion channel within which a magnesium binding site exists. At resting state, the ion channel is blocked by Mg$^{2+}$, which inhibits ion flow (Na$^+$, K$^+$ and Ca$^{2+}$) through the channel. This Mg$^{2+}$ block can be removed by depolarization of the plasma membrane [18]. NMDArs mediate long-term potentiation by allowing influx of Na$^+$, K$^+$ and Ca$^{2+}$ into the synapse. Excess activation of the NMDAr leads to excessive accumulation of intracellular Ca$^{2+}$ inducing apoptotic cascade cell death [19]. Other binding sites found on the NMDAr are the polyamine, zinc (NR2B) and proton sites [16].
Amongst the different binding sites on the NMDAr, those that reside inside the pore of the ion channel provide the opportunity for quantification of active, ‘open’ receptors. Non-competitive ion channel NMDAr antagonists, such as (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) and ketamine, gain access to the NMDAr ion channel binding site when the receptor is activated by its corresponding agonist glutamate and co-agonist glycine, i.e. these non-competitive antagonists are ‘use-dependent ligands’ [20,21]. Therefore, their binding is proportional to the number of activated, open receptor channels. It has been proposed that chronic activation of extrasynaptic NMDAr leads to a sustained neuronal amyloid-β release, which could be involved in the pathogenesis of Alzheimer’s disease [22-24]. Excessive activation of NMDArs may lead to neuronal death in a manner consistent with AD neuropathology [22,25]. Thus, using radiolabelled, non-competitive antagonists for the NMDAr ion channel and positron emission tomography (PET), the status of regional NMDAr activation can be assessed in vivo [26]. This could be of importance in studying the role of the NMDAr in neurological disorders, such as Alzheimer’s disease.

Today, 47 million people live with dementia worldwide of which Alzheimer’s disease is the most common form. It is estimated that 131 million people will suffer
from dementia in 2050 and that, worldwide, costs related to dementia will pass a trillion US dollar annually in 2018 [27]. One of its hallmarks is the deposition of amyloid β-peptide (Aβ) in plaques in the brain, causing neuroinflammation and neurodegeneration [28-31]. Those affect normal functioning of the brain and, in particular, neurotransmission in which the glutamatergic system plays an important role [32-34].

1.2. NMDA radiotracers

To date, more than 60 compounds have been radiolabelled and investigated for their suitability as a PET or single photon emission computed tomography (SPECT) tracer for the NMDAr. Those radiotracers are categorized into glycine ligands, NR2B ligands and ion channel ligands. Most radiotracers showed the same drawbacks when evaluated in vivo: poor brain penetration, extensive metabolism, high non-specific binding, homogenous brain distribution and/or a distribution that is inconsistent with the NMDAr distribution in the brain. Only seven ion channel tracers and one glycine site tracer were evaluated in human and they are summarized in Table 1 [19,35-40].

Table 1. Overview of NMDA radiotracers used in human applications and their main drawbacks.

<table>
<thead>
<tr>
<th>NMDA radiotracers</th>
<th>SPECT ligand</th>
<th>Ion channel</th>
<th>Uniform distribution</th>
<th>High non-specific binding due to lipohilicity</th>
<th>SPECT / PET ligand</th>
<th>Ion channel</th>
<th>High non-specific binding</th>
<th>Affinity for opiate receptors</th>
<th>Rapid appearance of labelled metabolites in plasma</th>
<th>Low contrast between regions of interest and reference regions</th>
<th>No significant difference between healthy human subjects and patients with temporal lobe epilepsy</th>
<th>Inter-subject variability</th>
<th>High perfusion dependent uptake</th>
<th>Specific binding not demonstrated</th>
<th>Brain distribution did not reflect the specific NMDA compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>[123I]iodo-MK-801([123I]1)</td>
<td>• SPECT ligand</td>
<td>• Ion channel</td>
<td>• Uniform distribution</td>
<td>• High non-specific binding due to lipohilicity</td>
<td></td>
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<td></td>
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<tr>
<td>[123/125I]CNS 1261 ([123/125I]2)</td>
<td>• SPECT / PET ligand</td>
<td>• Ion channel</td>
<td>• High non-specific binding</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>[11C]ketamine ([11C]3)</td>
<td>• PET ligand</td>
<td>• Ion channel</td>
<td>• Affinity for opiate receptors</td>
<td>• Rapid appearance of labelled metabolites in plasma</td>
<td>• Low contrast between regions of interest and reference regions</td>
<td>• No significant difference between healthy human subjects and patients with temporal lobe epilepsy</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>[18F]fluoromemantine ([18F]4)</td>
<td>• PET ligand</td>
<td>• Ion channel</td>
<td>• Inter-subject variability</td>
<td>• High perfusion dependent uptake</td>
<td>• Specific binding not demonstrated</td>
<td>• Brain distribution did not reflect the specific NMDA compartment</td>
<td></td>
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</tbody>
</table>
1.3. Positron Emission Tomography

PET imaging allows for the visualisation and quantification of the distribution of radiolabelled molecules in vivo at very low concentrations. In this manner, a whole range of physiological and molecular processes can be followed quantitatively at a regional tissue level.

PET is based on the principle of coincidence detection of two annihilation γ-photons of 511 KeV each. These photons originated from the annihilation of a positron (originating from a positron emitting nuclide) with an electron. As these two photons are emitted in almost opposite directions from each other, it is possible to identify the line along which this annihilation took place (line of response) using a PET scanner. By combining the information from all lines of response it is possible to reconstruct a 3D image of the distribution of radioactivity (Figure 2). Nowadays, PET scanners are combined with computed tomography (CT) or magnetic resonance imaging (MRI) to combine molecular information obtained using PET with anatomical information, making it an extremely powerful technique to study in real time molecular processes in vivo. This is important for the development of new diagnostic tools in the early detection of disease and in designing new drugs and even personalized medicines [41].
To image physiological and molecular processes, radiolabelled tracers are needed. These tracers are synthesised from a precursor and a radionuclide. Ideally, these radiolabelled tracers are structurally identical to their unlabelled counterparts. For radiolabelling, several radionuclides, such as $^{11}$C, $^{13}$N, $^{15}$O and $^{18}$F, are suitable (Table 2). Favourable isotopes have a high percentage of $\beta^+$ decay and a half-life that is suitable for the biological process to be followed. For most applications, $^{11}$C and $^{18}$F with half-lifes of 20 and 110 minutes, respectively, are the preferred radionuclides [40].

Table 2. Physical characteristics of the most commonly used positron emitters.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>$t_{1/2}$ (min)</th>
<th>$\beta^+$ decay (%)</th>
<th>Energy (KeV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{11}$C</td>
<td>20.4</td>
<td>99.8</td>
<td>960</td>
</tr>
<tr>
<td>$^{13}$N</td>
<td>9.97</td>
<td>100</td>
<td>1190</td>
</tr>
<tr>
<td>$^{15}$O</td>
<td>2.03</td>
<td>99.9</td>
<td>1720</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>110</td>
<td>97</td>
<td>635</td>
</tr>
</tbody>
</table>

1.4. Radiochemistry

The short half-lifes of the radionuclides used in the preparation of PET radiotracers sets limits to the total synthesis time, including purification, formulation and quality control. In general, the total synthesis time should not exceed 2 to 3 times the half-life of the radionuclide used. Therefore, the common strategy is to incorporate the radionuclide as late as possible in the synthesis sequence. Single step syntheses are most convenient and preferred. However, often multiple reaction steps are required in the synthesis of complex molecules. An example is radiolabelling via prosthetic groups and/or functional groups, which need to be deprotected after radiolabelling [40,42-44].
Radiochemistry differs from traditional synthetic chemistry in several ways. The amounts of radionuclides are extremely small, typically in the picomolar or nanomolar range. On the other hand, the amount of precursor usually is at the milligrams or micromole level. This excess compared with the radionuclide results in such high stoichiometric ratios that these reactions run at pseudo-first-order kinetics reaction rates. This, however, has also the advantage of increased reaction rates. Reactions that require hours or days under normal conditions can now be performed in minutes or even seconds. Typical reaction volumes are 0.2 – 1 mL and reaction temperatures may be up to 200 °C [40,42].

For radiolabelling with carbon-11 most synthetic routes are based on methylation of their corresponding desmethyl precursor. Methylation is carried out using [11C]methyl iodide or [11C]methyl triflate synthesised from cyclotron produced [11C]CO₂ or [11C]CH₄. Apart from this common route, there are many other possibilities for radiolabelling with carbon-11 using different [11C]synthons (Figure 3) [40,42,43].

![Figure 3. Most important [11C]synthons for radiolabelling, synthesised from either [11C]CO₂ or [11C]CH₄. Reprinted from [40] with permission.](image)

Fluorine-18 can be introduced directly into the target molecule, so called direct fluorination, or via indirect fluorination, using prosthetic groups. Direct fluorination can be performed using a single step reaction, but usually a second step is required to deprotect functional groups. Fluorination via the indirect approach always requires a multistep reaction. First, the radiolabelled prosthetic group needs to be synthesized, followed by a coupling reaction to the precursor and eventually a deprotection step [40,45,46].

Direct fluorination can be carried out using either electrophilic fluorination or nucleophilic fluorination. Electrophilic fluorination, where fluorine is obtained from the cyclotron as [18F]F₂, has one major drawback. The specific activity is rather low,
since during the synthesis of $^{[18F]}F_2$, carrier $^{[19F]}F_2$ gas needs to be added for recovery of $^{[18F]}F_2$ from the target. A different method to produce $^{[18F]}F_2$ is by reacting $^{[18F]}CH_3F$, prepared from $^{[18F]}F_{aq}$, with carrier $F_2$ in an inert neon matrix under electric discharges. Using this method higher specific activity can be achieved [47]. Nevertheless, both labelling methods produce radiopharmaceuticals with low to, at best, medium high specific activities. Electrophilically produced radiopharmaceuticals are therefore, in general, not suitable for PET investigations involving brain receptors [42,43].

Most often nucleophilic $^{18F}$-fluorination reactions are used to synthesise either $^{18F}$-fluorinated prosthetic groups or $^{18F}$-fluorinated radiopharmaceuticals. $^{[18F]}$fluorine is obtained as aqueous $^{18F}$- from the cyclotron. Fluoride ion in water is a poor nucleophile and inactive. The removal of water is crucial to improve nucleophilicity. The $^{18F}$ is trapped on an ion-exchange column and eluted using a basic solution, most commonly using potassium carbonate and a cryptand (Kryptofix 222) dissolved in a mixture of acetonitrile and water or tetra-n-butylammonium hydrogen carbonate solution. The water from the solution is then removed by evaporation. Nucleophilic fluorinations are usually performed in dipolar aprotic solvents. Aliphatic nucleophilic reactions proceed via an $S_{N}2$ mechanism. As leaving groups halogens and sulfonate groups are preferred. Aromatic nucleophilic substitutions are only possible when the aromatic ring is activated by (strong) electron-withdrawing groups on the ortho or para position, although this is not always necessary [40,42,43].

**1.5. Aim and outline**

As NMDAr radiotracers that are presently available suffer from several limitations, the aim of the work described in this thesis was to develop PET tracers for the NMDAr with improved in vivo characteristics.

This development involves lead identification, synthesis, structure activity relationships, radiosynthesis with either carbon-11 or fluorine-18, and finally preclinical evaluation in vivo. Chapter 2 describes the synthesis, structure activity relationship, radiolabelling and clinical evaluation in mice of high affinity ligands, based on $^{[11C]}$GMOM, for the ion channel of the NMDAr. Chapter 3 describes the synthesis, radiolabelling, structure activity relationship and evaluation of novel amine guanidine derivatives for the ion channel of the NMDAr in Wistar rats. In Chapter 4 a strategy is investigated to improve metabolic stability of $^{[11C]}$GMOM, which is evaluated in Wistar rats. Chapter 5 describes further evaluation of $^{[18F]}$PK-209, the most promising ligand emerging from Chapter 2, using PET in rhesus monkeys. Chapter 6 focuses on a compound that targets the NR2B site of the NMDAr, describing
its radiolabelling and evaluation in mice. Finally, in Chapter 7 a summary of Chapters 2 to 6 is provided together with a general discussion and future perspectives.
1.6. References


Synthesis, structure activity relationship, radiolabeling and preclinical evaluation of high affinity ligands for the ion channel of the N-methyl-D-aspartate receptor as potential imaging probes for positron emission tomography

Pieter J Klein, Johannes AM Christiaans, Athanasios Metaxas, Robert C Schuit, Adriaan A Lammertsma, Bart NM van Berckel, Albert D Windhorst

Published in: *Bioorganic & Medicinal Chemistry* 2015;23:1189-1206
Abstract

The N-Methyl-D-Aspartate receptor (NMDAr) is involved in many neurological and psychiatric disorders including Alzheimer’s disease and schizophrenia. Currently, it is not possible to assess NMDAr availability in vivo. The purpose of this study was to develop a positron emission tomography (PET) ligand for the NMDAr ion channel. A series of di- and tri-N-substituted diarylguanidines was synthesized. In addition, in vitro binding affinity for the NMDAr ion channel in rat forebrain membrane fractions was assessed. Compounds 15, 16 and 37 were radiolabeled with either carbon-11 or fluorine-18. Ligands [11C]15 and [18F]37 were evaluated ex vivo in B6C3 mice. Biodistribution studies showed higher uptake of [11C]15 and [18F]37 in forebrain regions compared with cerebellum. In addition, for [11C]15 54% and for [18F]37 70% of activity in the brain at 60 min was due to intact tracer. Pre-treatment with MK-801 (0.6 mg·kg⁻¹, i.p.) slightly decreased uptake in NMDAr-specific regions for [18F]37, but not for [11C]15. As such [18F]37 has the best characteristics as a PET tracer for the ion channel of the NMDAr.
2.1. Introduction

Glutamate plays an important role in neurotransmission in the brain via metabotropic and ionotropic receptors and it is the principal excitatory neurotransmitter [1]. The N-methyl-D-aspartate receptor (NMDAr) complex is one of the members of the ionotropic glutamate receptor family [2,3]. The NMDAr is involved in many neurological and psychiatric disorders including Alzheimer’s disease and schizophrenia. For instance, it has been proposed that chronic activation of extrasynaptic NMDAr leads to a sustained neuronal amyloid-β release, which could be involved in the pathogenesis of Alzheimer’s disease [4]. At present, it is not possible to assess NMDAr availability in vivo. Imaging of NMDAr using positron emission tomography (PET) could make this possible but requires the development of a suitable PET ligand.

NMDArs are heterotetrameric complexes of associated subunits [5]. Each NMDA ion channel is formed by the assembly of an obligatory glycine-binding NR-1 subunit, along with combinations of distinct glutamate-binding NR-2 (A-D) and/or glycine binding NR-3 subunits [6,7,8]. Amongst the different binding sites on the NMDAr, those that reside inside the pore of the ion channel provide the opportunity for quantification of active, ‘open’ receptors. Non-competitive ion channel NMDAr antagonists, such as (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) and ketamine, gain access to the NMDAr ion channel binding site when the receptor is activated by its corresponding agonist glutamate and co- agonist glycine, i.e. these non-competitive antagonists are ‘use-dependent ligands’ [9,10]. Therefore, their binding is proportional to the amount of activated, open receptor channels. Thus, using radiolabeled non-competitive antagonists for the NMDAr ion channel and PET, the status of regional NMDAr activation can be assessed in vivo.

The first study of N,N'-diarylguanidine affinity for the ion channel of the NMDAr complex was reported by Keana [11]. Since then, numerous compounds have been reported with NMDAr ion channel affinity, some of which showed high affinity in the nanomolar range and selectivity to the ion channel over sigma receptors (Figure 1), such as N-(1-naphthyl)-N’-3-iodophenyl)-N’-methylguanidine (CNS 1261, 2, Kᵦ: 4.21 nM against [³H]MK-801), which has been labeled with either iodine-123 ([¹²³I]2) [12] or iodine-125 ([¹²⁵I]2) [13], for single-photon emission computed tomography (SPECT).

N-(2-chloro-5-(thiomethyl)phenyl)-N’-(3-(thiomethyl)phenyl)-N’-methylguanidine (CNS 5161, 6, Kᵦ: 1.87 nM against [³H]MK-801) [14,15] and N-(2-chloro-5-(thiomethyl)phenyl)-N’-(3-(methoxy)phenyl)-N’-methylguanidine (GMOM, 5, Kᵦ: 5.20 nM against [³H]MK-801) [16,17] both labeled with carbon-11 are the most promising
NMDAr ion channel pore tracers to date, though clinical research applications have had limited success [18].

In humans, the highest uptake of $[^{123}\text{I}]2$ was found in thalamus followed by striatum, cortical regions and the lowest in white matter, which correlates with the NMDAr distribution [19]. A challenge with ketamine showed that $[^{123}\text{I}]2$ is a specific ligand for the ion channel of the NMDAr and that it can be displaced [20,21]. However, $[^{123}\text{I}]2$ revealed high nonspecific binding, limiting its potential for quantification as small changes in receptor availability are unlikely to be detected [12].

Heterogeneous distribution with lowest uptake in cerebellum and highest in putamen and thalamus was found in human using $[^{11}\text{C}]6$. Specific binding, however, has not been demonstrated and metabolism was rapid [22,23]. Ahmed et al found no significant increase in the uptake of $[^{11}\text{C}]6$ in patients with Parkinson’s disease compared with control subjects [15].

Using regional brain distribution and blocking studies in conscious rats, Waterhouse et al showed that $[^{11}\text{C}]5$ had a relatively low degree of saturable binding in vivo. In addition, $[^{11}\text{C}]5$ showed fast kinetics with high initial brain uptake followed by rapid washout in all regions examined. Pretreatment with unlabelled 3 or MK-801 induced a moderate and uniform decrease of $[^{11}\text{C}]5$ in most regions. However, no significant differences in regional ratios were found between blocked and control animals. The glycine-site agonist D-serine increased $[^{11}\text{C}]5$ uptake, while the NR2B modulator RO 25-6981 reduced the uptake of $[^{11}\text{C}]5$ in all regions, except for the striatum. PET studies in isoflurane anesthetized baboons provided a fairly uniform regional brain distribution, and blocking with MK-801 did not significantly alter regional values, indicating a lack of saturable binding [17].

Although a lot of effort has been put into the synthesis of $N,N'$diarylguanidines only two aromatic unsubstituted compounds were synthesized with a methylene spacer between the guanidine moiety and the aromatic ring, 1,3-dibenzylguanidine (IC$_{50}$: 6800 nM against $[^{3}\text{H}]$-N-[l-(2-thienyl)cyclohexyl]piperidine ($[^{3}\text{H}]$TCP)) [24] and 3-benzyl-1-methyl-1-(naphthal-1-yl)guanidine (IC$_{50}$: 2900 nM against $[^{3}\text{H}]$MK-801) [25].

The aim of this study was to synthesize and evaluate analogues of 5 as potential PET ligands for the NMDAr ion channel. Previous structure activity relationship (SAR)
Synthesis, structure activity relationship, radiolabeling and preclinical evaluation of high affinity ligands for the ion channel of the N-methyl-D-aspartate receptor as potential imaging probes for positron emission tomography

Studies showed that a meta substituted aryl group ($R_1$) (Figure 2) with small alkyl groups is favorable. Furthermore, on the other aryl group a 2,5-disubstitution pattern ($R_4$, $R_5$) indicated enhancement of potency, 2-bromo or 2-chloro combined with a 5-thiomethyl group showed the highest binding affinity [11,14,16,25]. Therefore, in this study a series of diarylguanidines was synthesized according to the structure in Figure 2 with phenyl or 3-methoxyphenyl as $R_1$ and $n = 0-2$ methylene groups. A second series of compounds consisted of the first series with a methyl moiety substituted at the $R_2$ position. The third library of compounds consisted of analogues of 5 substituted at the 3-methoxy moiety with short fluoroalkoxy chains, in addition 2 compounds of this series were methylated at the $R_3$ position instead of $R_2$. Synthesis, binding affinity to the ion channel of the NMDAR, radiolabeling of the high affinity compounds, LogD$_{7.4}$ value and biodistribution in mice were investigated.

Figure 2. General structure of N,N'-di- and tri-substituted arylguanidines.

2.2. Results

2.2.1. Chemistry

The disubstituted- and trisubstituted guanidines 5-27 (Scheme 1) were obtained via a method adapted from Hu et al [14] by reacting the appropriate cyanamides 29a-j and 30a-j with an amine hydrochloric salt in chlorobenzene. Products were collected as free base, and fumarate or hydrochloric acid salts.

The cyanamides 29a-j were prepared from commercially available primary amines 28a-j by reaction with cyanogen bromide in diethyl ether (29-48% yield). Subsequent alkylation with methyl iodide in presence of potassium carbonate in dimethylformamide yielded the N-methylcyanimides 30a-j (62-99%). N-(3-hydroxyphenyl)-N-methylcyanamide (30g), however, was synthesized using 1 equivalent of both potassium carbonate and methyl iodide instead of 1.1 and 2.0 equivalents to prevent O-methylation (65%) [26].

2-Chloro-N-methyl-5-(methylthio)aniline hydrochloride salt (81%) (34) (Scheme 2) was synthesized from 2-chloro-5-(methylthio)aniline (33), which was prepared in 2 steps by a modified Curtius rearrangement [27] of 2-chloro-(5-thiomethyl)-benzoic acid (31) (72%), with n-butyllithium as base and methyl iodide in THF [28].
t-butylcarbamate cyanamide (35) was prepared from 32 in 70% yield. The trifluoroacetyl protected aniline (36) was obtained quantitatively from aniline 33.

Scheme 1a. General synthesis scheme of guanidines.

\[
\text{ArN(CH}_3\text{)CN} \xrightarrow{a} \text{ArNH}_2 \xrightarrow{b} \text{ArNHCN} \xrightarrow{c} \text{Ar'NHAr'} \xrightarrow{d} \text{Ar'NHAr'} \xrightarrow{e} \text{Ar'NHAr'}
\]

- aR=H, n=0
- bR=H, n=1
- cR=H, n=2
- dR=OCH₃, n=0
- eR=OCH₃, n=1
- f R=OCH₃, n=2

\[9R=\text{OH}, n=0 \quad \text{9H}=\text{OCHF}_2, n=0 \quad \text{9I}=\text{OCF}_3, n=0 \quad \text{9J}=\text{OCF}_2\text{CHF}_2, n=0\]


\[
\text{HO}_2\text{Cl} \xrightarrow{a} \text{Boc'}\text{N} \xrightarrow{b} \text{H}_2\text{N} \xrightarrow{c} \text{Cl} \xrightarrow{d} \text{Boc'}\text{N} \xrightarrow{e} \text{F}_2\text{N} \xrightarrow{f} \text{F}_2\text{N}
\]

- aEt₃N, diphenyl phosphorazidate, t-BuOH, reflux
- b HCl, water, THF, reflux (72%, 2 steps, from 31)
- c n-BuLi 1.6M in hexanes, CH₃I, THF, 0 °C - rt (81%)
- d CNBr, NaH, THF, 0 °C – rt, (70%)
- e TFAA, Et₃N, CH₂Cl₂, 0 °C – rt, (94%)

The \(N,N'\)-disubstituted guanidines 18-25 (Scheme 1) were obtained by reaction of cyanamide 29a-j with 33 (25-84%). The \(N,N',N'\)-trisubstituted guanidines 26-27 (Scheme 1) were obtained from the reaction of the \(N\)-methycyanamide 29h,i with 34 (43-59%). Finally the reaction between \(N\)-methycyanamide 30a-j and 33 provided
the \(N,N,N'\)-trisubstituted guanidines 5,9-17 (36-78%) (Scheme 1). \(N,N',N'\)-Trisubstituted guanidines 37-39 were prepared by alkylation of 14 with appropriate bromo-fluoroalkane in 61-73% yield (Scheme 3).

Scheme 3a. Synthesis of fluoroalkoxy analogues.

\[
\text{HO-}N-N-N\text{Cl} \xrightarrow{a} \text{O-}N-N-N\text{Cl}
\]

\(a\) Reagents and conditions: (a) 37: Br\(\text{CH}_2\text{F}\) 2M in DMF, NaH, NaI, dimethoxyethane, rt (68%); 38: Br(\(\text{CH}_2\)\(\text{F}\)), K\(_2\)CO\(_3\), KI, DMF, 75 °C (61%); 39: Br(\(\text{CH}_2\)\(\text{F}\)), K\(_2\)CO\(_3\), KI, DMF, 75 °C (73%).

2.2.2. In vitro binding studies

The binding affinity of the ligands (5,9-27, 37-39) for the NMDAr channel was determined by measuring the ability of various concentrations of unlabeled ligand to inhibit specific binding of [\(^3\)H]MK-801 to rat forebrain membranes (P2 fraction). Results are shown in Table 1, with \(K_i\) (mean ± SD) values of 2-6 independent determinations, each conducted in triplicate.

Table 1. Binding affinity to the ion channel of the NMDAr against [\(^3\)H]MK-801.

<table>
<thead>
<tr>
<th>(R_1)</th>
<th>(R_2) = H, (R_3) = H</th>
<th>(R_2) = CH(_3), (R_3) = H</th>
<th>(R_2) = H, (R_3) = CH(_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{H})</td>
<td>18(^a) 722 ± 166</td>
<td>9(^b) 307 ± 130</td>
<td></td>
</tr>
<tr>
<td>(\text{H})</td>
<td>19(^c) &gt; 10 µM</td>
<td>10(^b) &gt; 10 µM</td>
<td></td>
</tr>
<tr>
<td>(\text{H})</td>
<td>20(^c) &gt; 10 mM</td>
<td>11(^c) &gt; 10 mM</td>
<td></td>
</tr>
<tr>
<td>(\text{OCH}_3)</td>
<td>21(^b) 136 ± 2.95</td>
<td>5(^b) 21.7 ± 7.52</td>
<td></td>
</tr>
<tr>
<td>(\text{OCH}_3)</td>
<td>22(^d) &gt; 1 µM</td>
<td>12(^d) &gt; 1 µM</td>
<td></td>
</tr>
<tr>
<td>(\text{OCH}_3)</td>
<td>23(^c) &gt; 10 µM</td>
<td>13(^c) &gt; 10 µM</td>
<td></td>
</tr>
<tr>
<td>(\text{OH})</td>
<td>114(^c) 551 ± 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{OCH}_2\text{F})</td>
<td>37(^b) 18.4 ± 8.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{OCHF}_2)</td>
<td>24(^c) 147 ± 25.3</td>
<td>15(^b) 10.2 ± 3.08</td>
<td>26(^b) 322 ± 33.7</td>
</tr>
<tr>
<td>(\text{OCF}_3)</td>
<td>25(^c) 235 ± 96.2</td>
<td>16(^c) 11.7 ± 4.16</td>
<td>27(^c) 650 ± 174</td>
</tr>
<tr>
<td>0((\text{CH}_2)(\text{F}))</td>
<td>38(^b) 155 ± 59.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0((\text{CH}_2)(\text{F})(\text{F}))</td>
<td>39(^b) 179 ± 66.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0((\text{OCF}_2)(\text{CH}_2)(\text{F}))</td>
<td>17(^b) 56.7 ± 10.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) \(K_i\) measured against 5 nM [\(^3\)H]MK-801 presented as mean ± SD of 2-6 independent determinations, each conducted in triplicate, \(^b\) fumaric acid salt, \(^c\) free base, \(^d\) hydrochloric acid salt.
2.2.3. Radiochemistry

The \(N,N'\)-[\(^{11}\)C]methyl-\(N'\)-tri substituted guanidine [\(^{11}\)C]15 (Scheme 4) was obtained through alkylation of \(N,N'\)-diarylguanidine 24 with [\(^{11}\)C]CH\(_3\)I although the unwanted side product \(N,N'\)-[\(^{11}\)C]methyl-\(N'\)-tri substituted guanidine ([\(^{11}\)C]26) was formed as well. Several base solvent combinations were explored (Table 2). Finally, [\(^{11}\)C]15 was synthesized starting from the precursor (24) in the presence of 5M sodium hydroxide in \(N,N'\)-dimethylformamide (Entry 2, Table 2). After preparative HPLC purification (Figure S1, Supplementary data), and formulating into a sterile solution, [\(^{11}\)C]15 was obtained in 26 ± 5\% (n=4) radiochemical yield, (decay corrected (DC), calculated from [\(^{11}\)C]CH\(_3\)I) and a radiochemical purity higher than 99.7\%. Specific activity was 170 ± 73.4 GBq·µmol\(^{-1}\) at end of synthesis (EOS) and total synthesis time, from end of bombardment (EOB), 38 min.

Scheme 4*. Radiosynthesis of [\(^{11}\)C]15 and [\(^{11}\)C]16 using [\(^{11}\)C]CH\(_3\)I.

Table 2. Radiochemical yields of carbon-11 labeled compounds under various conditions at 80 °C (calculated from [\(^{11}\)C]CH\(_3\)I).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Prec.</th>
<th>Base</th>
<th>µmol base</th>
<th>Solvent</th>
<th>RCY(^b) [(^{11})C]15</th>
<th>RCY(^b) [(^{11})C]26</th>
<th>Ratio(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>NaH</td>
<td>50</td>
<td>MeCN</td>
<td>48.3 ± 3.6</td>
<td>15.2 ± 0.1</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>NaOH</td>
<td>25</td>
<td>DMF</td>
<td>49.2 ± 0.6</td>
<td>23.8 ± 0.0</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>TBAOH</td>
<td>5</td>
<td>DMF</td>
<td>9.4 ± 1.6</td>
<td>1.0 ± 0.4</td>
<td>9.4</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>KOH</td>
<td>178</td>
<td>DMSO</td>
<td>54.8 ± 1.0</td>
<td>25.0 ± 3.0</td>
<td>2.2</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>TBAOH</td>
<td>25</td>
<td>DMF</td>
<td>3.0 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>7.5</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>TBAOH</td>
<td>10</td>
<td>DMF</td>
<td>3.1 ± 1.3</td>
<td>0.1 ± 0.1</td>
<td>31.0</td>
</tr>
<tr>
<td>7(^f)</td>
<td>24</td>
<td>TBAOH</td>
<td>5</td>
<td>DMSO</td>
<td>0.8</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>8(^f)</td>
<td>24</td>
<td>NaOH</td>
<td>25</td>
<td>DMSO</td>
<td>28.6</td>
<td>2.5</td>
<td>11.4</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>NaH</td>
<td>50</td>
<td>MeCN</td>
<td>37.9 ± 1.3</td>
<td>16.0 ± 1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>NaOH</td>
<td>25</td>
<td>DMF</td>
<td>42.0 ± 0.4</td>
<td>18.7 ± 1.0</td>
<td>2.2</td>
</tr>
<tr>
<td>11</td>
<td>25</td>
<td>KOH</td>
<td>178</td>
<td>DMSO</td>
<td>47.4 ± 2.6</td>
<td>15.3 ± 1.5</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*Reagents and conditions: (a) \([^{11}\)C]CH\(_3\)I, 5M NaOH, DMF, 80 °C.

\(^{a}\) Precursor, (1.4 µmol), \(^{b}\) NaH (60% dispersion in mineral oil), NaOH (5M), TBAOH (55% solution), KOH (powdered), \(^{c}\) 300 µL, \(^{d}\) Radiochemical Yield (RCY) determined by HPLC using system B in % ± SD (n=2), \(^{e}\) ratio = product divided by side product, \(^{f}\) n=1
Compound $[^{11}\text{C}]16$ was synthesized in the same manner as $[^{11}\text{C}]15$ (Table 2, entry 10) starting from 25 in 15 ± 8% (n=2) radiochemical yield, (DC, calculated from $[^{11}\text{C}]\text{CH}_3\text{I}$) and a radiochemical purity higher than 98.7% (System B). The specific activity was 226 ± 141 GBq·µmol$^{-1}$ (EOS).

To prevent formation of the $[^{11}\text{C}]$methylated side products ($[^{11}\text{C}]26$ and $[^{11}\text{C}]27$), an attempt was made to synthesize the $N,N'$-protected-$N'$ precursors 40, 41 (Scheme 5, Table S1 Supplementary data), but this approach was unsuccessful.

Scheme 5a. Attempted synthesis of protected precursors 40 and 41.

$[^{18}\text{F}]$Fluoromethylbromide was prepared via the method of Iwata et al [29] and subsequently reacted with 14 in the presence of sodium hydride and potassium iodide in $N,N$-dimethylformamide (Scheme 6). After HPLC purification (Figure S2, Supplementary data, System E) and formulation into a sterile solution, $[^{18}\text{F}]37$ was obtained in a maximum of 22% radiochemical yield (DC, calculated from $[^{18}\text{F}]\text{F}$) and radiochemical purity higher than 98% (Figure S2, Supplementary data, System D). Specific activity was 52.7 ± 34.1 GBq·µmol$^{-1}$ (EOS) and total synthesis time, from EOB, 92 min. Radiochemical yield of $[^{18}\text{F}]37$ via the intermediate $[^{18}\text{F}]$fluoromethyltriflate was 22% (DC).

Scheme 6a. Radiosynthesis of $[^{18}\text{F}]37$.

$[^{18}\text{F}]$Fluoromethylbromide was prepared via the method of Iwata et al [29] and subsequently reacted with 14 in the presence of sodium hydride and potassium iodide in $N,N$-dimethylformamide (Scheme 6). After HPLC purification (Figure S2, Supplementary data, System E) and formulation into a sterile solution, $[^{18}\text{F}]37$ was obtained in a maximum of 22% radiochemical yield (DC, calculated from $[^{18}\text{F}]\text{F}$) and radiochemical purity higher than 98% (Figure S2, Supplementary data, System D). Specific activity was 52.7 ± 34.1 GBq·µmol$^{-1}$ (EOS) and total synthesis time, from EOB, 92 min. Radiochemical yield of $[^{18}\text{F}]37$ via the intermediate $[^{18}\text{F}]$fluoromethyltriflate was 22% (DC).

Scheme 6a. Radiosynthesis of $[^{18}\text{F}]37$.

$[^{18}\text{F}]$Fluoromethylbromide was prepared via the method of Iwata et al [29] and subsequently reacted with 14 in the presence of sodium hydride and potassium iodide in $N,N$-dimethylformamide (Scheme 6). After HPLC purification (Figure S2, Supplementary data, System E) and formulation into a sterile solution, $[^{18}\text{F}]37$ was obtained in a maximum of 22% radiochemical yield (DC, calculated from $[^{18}\text{F}]\text{F}$) and radiochemical purity higher than 98% (Figure S2, Supplementary data, System D). Specific activity was 52.7 ± 34.1 GBq·µmol$^{-1}$ (EOS) and total synthesis time, from EOB, 92 min. Radiochemical yield of $[^{18}\text{F}]37$ via the intermediate $[^{18}\text{F}]$fluoromethyltriflate was 22% (DC).

Scheme 6a. Radiosynthesis of $[^{18}\text{F}]37$.  

$[^{18}\text{F}]$Fluoromethylbromide was prepared via the method of Iwata et al [29] and subsequently reacted with 14 in the presence of sodium hydride and potassium iodide in $N,N$-dimethylformamide (Scheme 6). After HPLC purification (Figure S2, Supplementary data, System E) and formulation into a sterile solution, $[^{18}\text{F}]37$ was obtained in a maximum of 22% radiochemical yield (DC, calculated from $[^{18}\text{F}]\text{F}$) and radiochemical purity higher than 98% (Figure S2, Supplementary data, System D). Specific activity was 52.7 ± 34.1 GBq·µmol$^{-1}$ (EOS) and total synthesis time, from EOB, 92 min. Radiochemical yield of $[^{18}\text{F}]37$ via the intermediate $[^{18}\text{F}]$fluoromethyltriflate was 22% (DC).

Scheme 6a. Radiosynthesis of $[^{18}\text{F}]37$.  

$[^{18}\text{F}]$Fluoromethylbromide was prepared via the method of Iwata et al [29] and subsequently reacted with 14 in the presence of sodium hydride and potassium iodide in $N,N$-dimethylformamide (Scheme 6). After HPLC purification (Figure S2, Supplementary data, System E) and formulation into a sterile solution, $[^{18}\text{F}]37$ was obtained in a maximum of 22% radiochemical yield (DC, calculated from $[^{18}\text{F}]\text{F}$) and radiochemical purity higher than 98% (Figure S2, Supplementary data, System D). Specific activity was 52.7 ± 34.1 GBq·µmol$^{-1}$ (EOS) and total synthesis time, from EOB, 92 min. Radiochemical yield of $[^{18}\text{F}]37$ via the intermediate $[^{18}\text{F}]$fluoromethyltriflate was 22% (DC).

Scheme 6a. Radiosynthesis of $[^{18}\text{F}]37$.  

$[^{18}\text{F}]$Fluoromethylbromide was prepared via the method of Iwata et al [29] and subsequently reacted with 14 in the presence of sodium hydride and potassium iodide in $N,N$-dimethylformamide (Scheme 6). After HPLC purification (Figure S2, Supplementary data, System E) and formulation into a sterile solution, $[^{18}\text{F}]37$ was obtained in a maximum of 22% radiochemical yield (DC, calculated from $[^{18}\text{F}]\text{F}$) and radiochemical purity higher than 98% (Figure S2, Supplementary data, System D). Specific activity was 52.7 ± 34.1 GBq·µmol$^{-1}$ (EOS) and total synthesis time, from EOB, 92 min. Radiochemical yield of $[^{18}\text{F}]37$ via the intermediate $[^{18}\text{F}]$fluoromethyltriflate was 22% (DC).

Scheme 6a. Radiosynthesis of $[^{18}\text{F}]37$.  

$[^{18}\text{F}]$Fluoromethylbromide was prepared via the method of Iwata et al [29] and subsequently reacted with 14 in the presence of sodium hydride and potassium iodide in $N,N$-dimethylformamide (Scheme 6). After HPLC purification (Figure S2, Supplementary data, System E) and formulation into a sterile solution, $[^{18}\text{F}]37$ was obtained in a maximum of 22% radiochemical yield (DC, calculated from $[^{18}\text{F}]\text{F}$) and radiochemical purity higher than 98% (Figure S2, Supplementary data, System D). Specific activity was 52.7 ± 34.1 GBq·µmol$^{-1}$ (EOS) and total synthesis time, from EOB, 92 min. Radiochemical yield of $[^{18}\text{F}]37$ via the intermediate $[^{18}\text{F}]$fluoromethyltriflate was 22% (DC).
2.2.4. Determination of lipophilicity expressed as LogD$_{oct,7.4}$ of 5, 15, 16 and 37

The distribution coefficients of 5, 15, 16 and 37 were measured using $[^{11}\text{C}]5$, $[^{11}\text{C}]15$, $[^{11}\text{C}]16$ and $[^{18}\text{F}]37$ [30]. The LogD$_{oct,7.4}$ values are shown in Table 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogD$_{oct,7.4}$a</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{11}\text{C}]5$</td>
<td>1.72 ± 0.01</td>
</tr>
<tr>
<td>$[^{11}\text{C}]15$</td>
<td>1.76 ± 0.01</td>
</tr>
<tr>
<td>$[^{11}\text{C}]16$</td>
<td>2.76 ± 0.01</td>
</tr>
<tr>
<td>$[^{18}\text{F}]37$</td>
<td>1.45 ± 0.02</td>
</tr>
</tbody>
</table>

a n=3, presented as mean ± SD.

2.2.5. Metabolite analysis

Metabolite analysis of $[^{11}\text{C}]15$ and $[^{18}\text{F}]37$ were determined in B6C3 mice at the following time points: 5, 15, 30, 60 min using 3 or 4 animals per time point. Table 4 shows the distribution of radioactivity amounts between polar and non-polar fraction. The non-polar fraction is divided into three fractions, parent compound, metabolites and remaining activity on the solid phase extraction (SPE) column.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Timea</th>
<th>n</th>
<th>Polar fraction</th>
<th>Non-polar fraction</th>
<th>SPEb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Parent</td>
<td>Metabolite</td>
<td>SPE</td>
</tr>
<tr>
<td>$[^{11}\text{C}]15$</td>
<td></td>
<td></td>
<td>74 (28)</td>
<td>20 (21)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Brain</td>
<td>5</td>
<td>3</td>
<td>73 (31)</td>
<td>21 (23)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>4</td>
<td>63 (20)</td>
<td>24 (14)</td>
<td>6 (9)</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>7</td>
<td>54 (19)</td>
<td>29 (10)</td>
<td>6 (6)</td>
</tr>
<tr>
<td>60</td>
<td>4</td>
<td>11</td>
<td>54 (19)</td>
<td>29 (10)</td>
<td>6 (6)</td>
</tr>
<tr>
<td>Plasma</td>
<td>5</td>
<td>4</td>
<td>39 (20)</td>
<td>43 (15)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>29</td>
<td>17 (3)</td>
<td>53 (13)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>25</td>
<td>14 (7)</td>
<td>61 (6)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>60</td>
<td>4</td>
<td>34</td>
<td>7 (2)</td>
<td>59 (6)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>$[^{18}\text{F}]37$</td>
<td></td>
<td></td>
<td>86 (9)</td>
<td>8 (9)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Brain</td>
<td>5</td>
<td>3</td>
<td>72 (17)</td>
<td>24 (20)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>2</td>
<td>76 (15)</td>
<td>18 (12)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>3</td>
<td>70 (16)</td>
<td>25 (14)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>60</td>
<td>4</td>
<td>4</td>
<td>38 (6)</td>
<td>48 (11)</td>
<td>7 (8)</td>
</tr>
<tr>
<td>Plasma</td>
<td>5</td>
<td>4</td>
<td>38 (6)</td>
<td>48 (11)</td>
<td>7 (8)</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>20</td>
<td>25 (6)</td>
<td>54 (8)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>16</td>
<td>21 (14)</td>
<td>59 (14)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>60</td>
<td>3</td>
<td>29</td>
<td>9 (4)</td>
<td>61 (3)</td>
<td>1 (0)</td>
</tr>
</tbody>
</table>

a time in min. b The remaining measured radio activity on the solid phase extraction (SPE) column.
2.2.6. *Ex vivo* biodistribution

Biodistribution and regional brain uptake of $[^{11}C]15$ and $[^{18}F]37$ were determined in anaesthetized B6C3 mice at the following time points: 5, 15, 30, 60 min.

Brain uptake of $[^{11}C]15$ was highest at 5 min when compared with all other time points (Figure 3; $p<0.001$, Least Significant Difference (LSD) posttests). Standardized uptake values (SUV) at 5 min were 0.70 ± 0.09 for total brain and 1.41 ± 0.46, 0.83 ± 0.11, 0.86 ± 0.13, 0.96 ± 0.16 and 0.55 ± 0.07, for hippocampus, cerebral cortex, striatum, prefrontal cortex and cerebellum, respectively. Total brain uptake was 0.32 ± 0.11, 0.27 ± 0.11 and 0.14 ± 0.07 at 15, 30 and 60 min. The highest ratio of radioactivity uptake between forebrain regions and cerebellum was observed at 5 min post injection. Radioactivity was cleared rapidly from the brain and no regional differences in uptake were observed 15 min after tracer injection. Two way Analysis of Variance (ANOVA) confirmed significant main effects of region [$F(4,15)=4.8$, $p<0.01$] and time [$F(3,45)=11.6$, $p<0.001$] on brain uptake of $[^{11}C]15$.

Organ uptake of $[^{11}C]15$ was higher in lungs and kidney than in other organs (Figure 3; $p<0.001$, LSD posttests). The highest $[^{11}C]15$ uptake was measured in the lungs, 5 min following tracer injection ($p<0.001$; LSD posttests), whereas the lowest levels of radioactivity were observed in blood. Organ uptake of $[^{11}C]15$ was higher at 5 min post injection than at the other time points measured ($p<0.001$; LSD posttests). Significantly higher levels of $[^{11}C]15$ uptake were observed in the kidney 60 min post-injection when compared with all other organs ($p<0.01$; LSD posttests). Two way ANOVA confirmed significant main effects of organ [$F(4,12)=130.1$, $p<0.001$] and time [$F(3,36)=24.7$, $p<0.001$] on uptake of $[^{11}C]15$, as well as significant organ x time interaction effects [$F(12,36)=8.3$, $p<0.001$].

![Figure 3. Biodistribution of $[^{11}C]15$ in the CNS (left) and selected organs (right).]
Chapter 2

Brain uptake of $[^{18}F]37$ was highest at 5 min when compared with all other time points (Figure 4; $p<0.01$, LSD posttests). SUV values at 5 min were 0.84 ± 0.12 for total brain and 1.05 ± 0.09, 1.17 ± 0.07, 1.19 ± 0.15, 1.19 ± 0.10 and 0.84 ± 0.09 for hippocampus, cerebral cortex, striatum, prefrontal cortex and cerebellum, respectively. Total brain uptake was 0.59 ± 0.04, 0.53 ± 0.07 and 0.23 ± 0.03 at 15, 30 and 60 min. $[^{18}F]32$ uptake was lower in cerebellum than in all other brain areas analyzed ($p<0.01$, LSD posttests). The highest ratio of radioactivity uptake between forebrain regions and cerebellum was observed at 15 min post injection. Radioactivity cleared rapidly from the brain and no regional differences in uptake were observed 60 min after tracer injection. Two way ANOVA confirmed significant main effects of region [$F(4,14)=4.3$, $p<0.01$] and time [$F(3,42)=49.9$, $p<0.001$] on brain uptake of $[^{18}F]37$.

Organ uptake of $[^{18}F]37$ was higher in lungs and kidney than in other organs (Figure 4; $p<0.001$, LSD posttests). The highest $[^{18}F]37$ uptake was measured in the lungs, 5 min following tracer injection ($p<0.001$; LSD posttests), whereas the lowest levels of radioactivity were observed in blood. Organ uptake of $[^{18}F]37$ was higher at 5 min post injection than at other time points ($p<0.001$; LSD posttests). Activity was rapidly cleared from the lungs, and only the kidneys showed significantly higher levels of $[^{18}F]37$ 60 min post injection than other organs ($p<0.01$; LSD posttests). Two way ANOVA confirmed significant main effects of organ [$F(4,15)=51.1$, $p<0.001$] and time [$F(3,45)=17.1$, $p<0.001$], as well as significant organ x time interaction effects [$F(12,45)=12.1$, $p<0.001$] on uptake of $[^{18}F]37$.

Figure 4. Biodistribution of $[^{18}F]37$ in the CNS (left) and selected organs (right).

2.2.7. Ex vivo autoradiography

Non specific binding was defined as activity that remained following pretreatment with MK-801 (0.6 mg·kg$^{-1}$, intraperitoneal injection). For $[^{11}C]15$, pretreatment
resulted in an increased (t-test, t(8)=6.3, P<0.001) uptake of $[^{14}C]15$ in striatum, frontal cortex, hippocampus, cerebral cortex and cerebellum (Figures 5 & S3, Supplementary data). For $[^{18}F]37$, pretreatment resulted in a decreased (t-test, t(8)=4.7, P<0.01) uptake of $[^{18}F]37$ in hippocampus, striatum, cerebral cortex, cerebellum and frontal cortex (Figures 5 & S4, Supplementary data).

Figure 5. Relative optical density (ROD) values of several brain areas in control and after pretreatment with 0.6 mg·kg$^{-1}$ MK-801. On the left uptake of $[^{14}C]15$, on the right uptake of $[^{18}F]37$, Data represent mean ± SEM of 2 ($[^{14}C]15$) or 3 ($[^{18}F]37$) determinations.

2.3. Discussion

In this study binding affinities to the NMDAr of a series of compounds, based on lead compound 5, were investigated. Compounds with high affinity were labeled with a positron emitter and subsequently evaluated preclinically as NMDAr uncompetitive antagonists. Waterhouse et al reported previously, for 5, a binding affinity for the NMDAr of 5.20 ± 0.3 nM [31], which is different from the present value of 21.7 ± 7.52 nM. This difference in $K_i$ values between the two studies could be due to differences in animals used (Sprague-Dawley rats vs. Wistar rats), differences in membrane preparation procedures (total membrane fraction vs. P2 fraction used here), different binding properties of NMDArs in the different brain regions used for obtaining membranes (frontal cortex vs. total forebrain used here) or even different assay conditions (i.e. time of incubation). The most striking difference between the two studies, however, is the amount of glutamate and glycine used in the assays. In the present study 1µM of glutamate and glycine was used to supplement basal binding conditions, and to obtain a known and constant concentration of these modulators. Waterhouse et al [31] used 10 mM of glutamate and glycine, which represents suprasaturating conditions. As the binding site within the channel of the NMDAr is only
accessible when the receptor is activated by its corresponding agonists, glutamate and glycine [9,10], the difference in K_i values between the two studies may be due to the amount of open channel receptors, induced by the use of different concentrations of glutamate and glycine in the two assays.

To confirm the in vitro binding result, 5 was screened commercially at Cerep (www.cerep.fr). The result obtained in the presence of 100 µM glutamate (no glycine) was 15 nM, and ties in with the value presented in this study.

2.3.1. Chemistry

The guanidines (3,9-27) were synthesized by reaction of the corresponding N-cyanamides (29a-j) or N-methylcyanamides (30a-j) with either the hydrochloric acid salt of 2-chloro-5-(methylthio)aniline (33) or 2-chloro-N-methyl-5-(methylthio)aniline (34) in yields comparable with those reported in literature [14,24,25]. Starting cyanamides were prepared by the reaction of the appropriate primary amines with cyanogenbromide in diethyl ether in yields of up to 48%. The short fluoroalkyl guanidines (37-39) were synthesized from the phenolic guanidine 14 by alkylation with F(CH2)nBr (n=1-3) with potassium carbonate and potassium iodide in DMF. Compounds 38 and 39 were obtained in a yield of 61 and 73%, respectively. For the synthesis of the fluoromethoxy analogue (37), however, this method was not applicable. Using sodium hydride and sodium iodide in dimethoxyethane gave 68% yield.

2.3.2. Structure Activity Relationships

Since only two compounds with a methylene spacer between the aromatic group (Figure 2) and the guanidine moiety have been described [24,25], little is known about SARs within this class of compounds. Introduction of one (19, 22, 10, 12) or two methylene groups (20, 23, 11, 13), reduced the binding affinity for the ion channel of the NMDAr dramatically with an order of 10^2 to more than 10^6 compared with 18, 21, 9, 5, indicating that elongation of the chain length is not tolerated for NMDAr binding.

Although the N-methyl group in the guanidine structure is present in all high affinity compounds reported [14,25], one cannot conclude from SAR studies that this moiety is responsible for the higher affinity for the ion channel. Nevertheless, a better selectivity over the sigma receptor compared with non-methylated guanidines has been reported [14,25].
Introducing a N-methyl group in compounds 18, 21, 24 and 25 increased binding affinities 2, 6, 14 and 20 fold for 9, 5, 15 and 16, respectively. A combination of the 3-methoxy moiety and the N-methyl group led to an increase in affinity to 21.7 ± 7.52 nM for 5 compared with 18 (722 ± 166 nM). In contrast, when methylated on the N'-guanidine position next to the 2-chloro-5-(methylthio)phenyl ring, the affinity of compounds 26 and 27 are decreased 2 to 3 fold compared with 24 and 25, respectively. This indicates that N-methylation leads towards improved affinity but N'-methylation is not accepted.

The need of a small alkyl group at the 3-position as described by Keana et al [11], Reddy et al [25], Hu et al [14] and Dumont et al [16] is supported by the results of R1 substitution of the aryl group with 3-OH or 3-methoxy. The binding affinity of the phenol 14 decreased approximately 2-fold compared with aryl 9 (307 ± 130 nM), while the methoxy analogue 5 showed a 14 fold increase of potency.

Substitution of hydrogen by fluorine may have a positive effect on physicochemical properties of ligands, such as binding affinity and CNS penetration [32]. In addition, the presence of a fluorine atom gives the opportunity of radiolabeling with fluorine-18 for use with PET, as exchange of fluorine-19 by fluorine-18 would not affect the physicochemical properties. However, this is only possible if the fluorine position is amenable to radiolabeling with fluorine-18.

Introduction of a monofluoromethoxy group in 37 (18.4 nM) was tolerated as the affinity towards the NMDAr was not significantly altered compared with 6 (21.7 nM). Additionally, substitution of one or two further hydrogen atoms by fluorine led to an increase to 10.2 nM for the difluoromethoxy guanidine (15) and 11.7 nM for the trifluoromethoxy guanidine (16), roughly a 2 fold increase in affinity. Substitution of the fluoromethoxy moiety of ligand 37 with 1-fluoroethoxy or 1-fluoropropoxy reduced binding affinity 8 fold for compound 38 (155 ± 59.0 nM) and 10-fold for 39 (179 ± 66.9 nM). Given the improved affinity of the trifluoromethoxy compared with the monofluoromethoxy compound, the 1,1,2,2-tetrafluoroethoxy analogue (17) was also synthesized. Compared with the 1-fluoroethoxy ligand (38) the affinity increased almost 3-fold to 56.7 ± 10.8 nM for 17. These results suggest that substitution of the alkoxy moiety with one or more fluorine atoms is beneficial for the binding affinity towards the NMDAr.


The carbon-11 labeling agent [11C]CH3I was prepared by the reaction of cyclotron produced [11C]CO2 with lithiumaluminiumhydride in THF, which subsequently was reacted with hydroiodic acid in radiochemical yields of up to 90% (DC). After
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[^11C]methylation of 24, two products were obtained, the desired[^11C]15 as the main product and the side product[^11C]26. Several base and solvent combinations were explored (Table 2, Entries 1-4). Three combinations resulted in radiolabeling yields between 48 and 55% for[^11C]15 and a ratio between main product and side product of 2.1 to 3.2. The use of t-butylammonium hydroxide (TBAOH) in combination with DMF lowered the yield (9%), although a highly increased ratio of 9.8 was observed. Given this ratio and the highest radiolabeling yields obtained with DMF and DMSO as solvents, the combination with TBAOH in different concentrations (Table 2, Entries 5-7) was carried out. Unfortunately, yields dropped to 3% or less, although even with a better ratio. Using DMSO and potassium hydroxide the highest yield (55%) was obtained, while using sodium hydroxide as base resulted in a yield of 29%. Modifications of reaction temperature and time were not investigated, as the yield of the radiolabeling was sufficient for preclinical evaluation.

Due to the isomeric products[^11C]15 and[^11C]26, semi-preparative HPLC purification is delicate. However, using a Platinum C18-EPS semi-preparative HPLC column, both isomers could easily be separated with a retention time of 6 min for the desired product and 21 min for the side product (Figure S1, Supplementary data).

During optimization of the synthesis of[^11C]16 starting from 25, the same pattern of labeling yield and formation of side product[^11C]27 was observed.[^11C]16 was obtained in yields of 38-47% (Table 2, Entries 9-11).

To prevent the formation of[^11C]26 and[^11C]27, an attempt was made to synthesize the N'-protected precursors 40 and 41 (Scheme 5). First, synthesis of the tert-butyloxycarbonyl (t-boc) protected precursor (40) was attempted, but this failed due to the acidic conditions during the condensation reaction of the guanidine. HCl in the reaction mixture eliminates the t-boc protective group (Table S1, Entries 1-2). Therefore, subsequent reactions were carried out with the free base. Guanidine formation in chlorobenzene had no success, using hexa-fluoro isopropanol (HFIP) 40 was obtained in 1% yield (Table S1, Entry 3, Supplementary data) [33,34]. Also, the reaction of t-boc (32) or trifluoroacetyl protected (36) aniline with cyanamide 29h in either chlorobenzene or HFIP did not yield the desired N,N'-protected,N' precursors 40 and 41 (Table S1, Entries 4-6, Supplementary data). Therefore, the synthesis of[^11C]15 and[^11C]16 was carried out with the unprotected precursors 24 and 25.

2.3.4. Synthesis of[^18F]37

The synthesis of[^18F]37 was carried via a two-step synthesis starting from[^18F]fluorine. First, [^18F]fluoromethylbromide was prepared, distilled and purified according to the method of Iwata et al [29] in comparable yields of 37-74% (DC).
Secondly, it was reacted with the phenol precursor 14 in DMF with potassium carbonate and potassium iodide as catalysts, in yields up to 8% (DC) overall of [18F]37. Changing the base to sodium hydride, however, improved the yield of [18F]37 to 22% (DC) overall. Iwata et al also reported an improvement of alkylation when the intermediate [18F]fluoromethylbromide was converted online to [18F]fluoromethyltriflate via a heated AgOTf column [29]. This method was also investigated, but yields were not higher than those using [18F]fluoromethylbromide.

2.3.5. Distribution coefficients

Measured LogD oct,7.4 values of 5, 15, 16 and 37 are shown in Table 3. According to Waterhouse and Pike only 16 (2.76) is within the optimum range of 2.0 - 3.5 [35,36]. However, both [11C]15 and [18F]37 enter the brain (Figures 3 & 4), with LogD values of 1.76 and 1.45, respectively.

2.3.6. Biological evaluation

Given the comparable affinity of 15 and 16 (10.2 and 11.7 nM respectively), only the carbon-11 labeled compound [11C]15 was evaluated in B6C3 mice with respect to metabolite formation and biodistribution in addition to the fluorine-18 labeled ligand [18F]37.

2.3.7. Metabolites of [11C]15 and [18F]37

For PET it is important to consider metabolism of a radiolabeled compound in vivo. The presence of radiolabeled metabolites may hamper quantification of PET images in humans since PET cannot distinguish between signals from parent radiolabeled ligand and its radiolabeled metabolites [37].

After 60 minutes, 54 ± 19% of the measured radioactivity in the brain was due to parent compound ([11C]15), whilst the remaining radioactivity accounted for polar (11 ± 5%) and non-polar (29 ± 10%) metabolites. In plasma, however, only a parent fraction of 7 ± 2% was measured, 34 ± 7% was due to polar metabolites and 59 ± 6% to non-polar metabolites.

HPLC analysis revealed three radiolabeled non-polar metabolites in the brain, which were more polar than the parent compound. Most likely, these metabolites are the result of cleavage of the S-methyl or CHF2 moiety. Cleavage of the [11C]N-methyl moiety of [11C]15 could lead to polar metabolites, most likely representing single
carbon molecules such as $[^{11}\text{C}]$formaldehyde, $[^{11}\text{C}]$formic acid or $[^{11}\text{C}]$carbon dioxide and an unlabelled metabolite of 15.

The parent compound $[^{18}\text{F}]37$ accounted for $70 \pm 6\%$ of the measured radioactivity in the brain after 60 minutes, with $25 \pm 14\%$ non-polar and $4 \pm 1\%$ polar metabolites, respectively. On the other hand, the parent fraction in plasma was only $9 \pm 4\%$, whereas $61 \pm 3\%$ and $29 \pm 0\%$ was due to non-polar and polar metabolites, respectively.

The three radiolabeled non-polar metabolites of $[^{18}\text{F}]37$, measured using HPLC, were more polar than the parent compound $[^{18}\text{F}]37$ and could be the result of cleavage of the $N$-methyl or $S$-methyl moieties. Polar metabolites are probably due to cleavage of the $[^{18}\text{F}]\text{CH}_2\text{F}$ moiety. It has been reported that substitution of the fluoromethoxy moiety by a fluorodideuteromethoxy group can prevent cleavage of this moiety [38]. This needs to be investigated in future studies. Defluorination, giving $[^{18}\text{F}]$fluoride ion which binds rapidly to bone, is not observed as the activity in the skull remains the same over time.

For quantitative PET imaging the amount of intact parent compound is the most important factor. Radiolabeled metabolites are nearly always present in plasma after tracer administration, and their presence can be accounted for by obtaining plasma samples and quantifying the parent fraction. Radiolabeled metabolites present in the brain, on the other hand, may prevent the possibility to model the tracer and to derive quantitative parameters.

If the rate and profile of metabolite formation remains identical between rodents and humans, then our results in mice might preclude the possibility to successfully model the uptake of $[^{18}\text{F}]37$ in subsequent human PET studies. It should be noted, however, that extrapolating profiles of metabolite formation across species is likely to be tenuous, and that there are well-documented differences, both in the rate and in the species of metabolite formation between rodents and humans [36,39]. Hence, experiments in higher species are required to unequivocally clarify whether the metabolic profile of $[^{18}\text{F}]37$ will preclude the use of the radiotracer for quantifying NMDAr activation using PET.

The range of standard deviations in these types of experiments can vary a lot. This can be caused by a combination of factors such as $\textit{in vivo}$ variation, work-up procedures and time pressure due to the short half-life time of carbon-11 (20 min) or fluorine-18 (110 min). The remaining measured radioactivity on the solid phase extraction (SPE) column is typically assumed to be parent compound as it is the most apolar compound.
2.3.8. Biodistribution and blocking

For both radiolabeled ligands, regional uptake patterns are in line with the distribution of NMDAr in the brain [40,41]. The highest uptake was observed in hippocampus and the lowest in cerebellum, with moderate levels in cerebral cortex, striatum and frontal cortex.

*Ex vivo* autoradiography was performed at 5 minutes for $^{[11]C}15$, and at 15 minutes for $^{[18]F}37$ post injection as the biodistribution showed the highest uptake in the hippocampus for both compounds (Figure S3 & S4, Supplementary data). Data from rodent studies indicate that 50% occupancy of the NMDAr ion-channel by MK-801 is obtained at a dose of 0.18 mg/kg, *in vivo* [42,43]. In this study we used 0.6 mg·kg$^{-1}$ MK-801, which is at least three times the *in vivo* EC$_{50}$ to cover the majority of NMDArs and give us a specific signal.

It showed that uptake of $^{[11]C}15$ was increased by pretreatment of MK-801. The reason for this is unclear, and we have not further investigated it.

In contrast, *ex vivo* autoradiography suggests that the uptake of $^{[18]F}37$ is selective for the NMDAr, as uptake decreased following pretreatment with a pharmacological dose of MK-801. Though the structural difference between $^{[11]C}15$ and $^{[18]F}37$ is only one fluorine atom, it seems that this can have strong effects on the binding characteristics of a ligand.

One-to-one comparison of $^{[18]F}37$ with $^{[11]C}5$ in non-human primates would be the next step. However, as reviewed by Waterhouse *et al* one should take care of using non-human primates for studying the NMDA receptor as it is vulnerable for anesthetics [44]. In that view PET imaging in human should be the next step of development of this new tracer.

2.4. Conclusion

Structural modification of 5 led to three new ligands (15, 16 and 37) with increased affinity for the ion channel of the NMDA receptor. Ligands $^{[11]C}15$ and $^{[18]F}37$ were evaluated by *ex vivo* biodistribution in mice and showed rapid metabolism in plasma, however, in the brain much less metabolites were observed. After pretreatment with a relatively low dose of the selective NMDAr antagonist MK-801, $^{[18]F}37$ showed slightly reduced brain uptake. These results suggest that $^{[18]F}37$ has promise as a PET tracer for the NMDA receptor.
2.5. Experiments

2.5.1. Chemistry

Reagents and solvents were supplied by Aldrich Chemical Co., Fluka, ABCR, Acros or Biosolve BV, and used as received unless stated otherwise. Nuclear magnetic resonance (NMR) spectra (\textsuperscript{1}H, \textsuperscript{13}C, \textsuperscript{19}F NMR) were recorded on a Bruker Avance 250 (250.13, 62.90, 235.36 MHz, respectively). Chemical shifts of the NMR spectra are reported in parts per million (ppm) relative to the solvent residual peak. Description of signals: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, ddd = doublet of dd, dt = doublet of triplets, tt = triplet of triplets, dq = double quartet. Thin-layer chromatography (TLC) was performed on Merck DC-alufolien, silica gel 60, F254. Flash column chromatography was performed on silica gel 60Å, 230 – 400 Mesh. The HPLC analysis system consisted of a Jasco PU-2089 HPLC pump, a Rheodyne injector with a 20 μL loop, a Jasco UV-2075 Plus UV detector set at a wavelength of 254 nm and a Raytest Na(I) radioactivity detector. HPLC data were collected and integrated using the software package GINA 5.01. HPLC: System A: Alltech Platinum EPS, C18 100A 5μ, 250 x 4.6 mm column using 50 mM NH₄H₂PO₄ pH=2.5 / methanol (30/70, v/v) as eluent and a flow rate of 1 mL⋅min\(^{-1}\); System B: Waters µBondapak, C18 10μm 125Å, 250 x 4.6 mm column using 10 mM NH₄HCO₃ pH=7.4 / acetonitrile (30/70, v/v) as eluent and a flow rate of 1.5 mL⋅min\(^{-1}\); System C: Alltech Platinum EPS, C18 100A 5μ 150 x 10.0 mm column using 10 mM NH₄HCO₃ pH=7.4 / acetonitrile (10/90, v/v) as eluent and a flow rate of 5 mL⋅min\(^{-1}\); System D: Alltech GraceSmart C18, 5μ, 250 x 4.6 mm column using 10 mM NH₄H₂PO₄ pH=2.5 / acetonitrile (75/25, v/v) as eluent and a flow rate of 1.5 mL⋅min\(^{-1}\); System E: Alltech Alltima C18, 5μ, 250 x 10.0 mm column using 25 mM NH₄H₂PO₄ pH=2.5 / acetonitrile (72/28, v/v) as eluent and a flow rate of 5.0 mL⋅min\(^{-1}\); System F: Dionex Ultimate 3000 HPLC system, equipped with a Phenomenex Gemini C18 5μ 250 x 10.0 mm column using a gradient of 50 mM NH₄H₂PO₄ / acetonitrile (80/20 to 30/70, v/v) in 12.5 minutes at a flow rate of 3.0 mL⋅min\(^{-1}\). For high-resolution mass spectrometry (HRMS), a Finnigan MAT 90 was used. Mass spectra (MS) were measured on an AB Sciex Qtrap 5500. [\textsuperscript{14}C]CO\(_2\) was produced by the \textsuperscript{14}N(p,α)\textsuperscript{11}C nuclear reaction and [\textsuperscript{18}F]F\(^-\) was produced by the \textsuperscript{18}O(p,n)\textsuperscript{19}F nuclear reaction using an IBA Cyclone 18/9 cyclotron. Radioactivity levels were measured using a Veenstra VDC-405 dose calibrator. Radiochemistry was carried out in homemade, remotely controlled devices [45].
2.5.1.1. General Procedure for N-Cyanation (A).

A solution of cyanic bromide [Caution, highly toxic] (2 equiv.) in diethyl ether (10 mL) was added dropwise to a solution of the appropriately substituted aniline (1 equiv.) in diethyl ether (10 mL) at 0 °C. After complete addition the mixture was warmed to ambient temperature and stirred 1-20 hours, which was followed by TLC. Solids were filtrated and washed with ether. The filtrate was washed with 1M HCl (25 mL) followed by brine (25 mL). The organic layer was collected, dried over anhydrous MgSO₄, filtrated and evaporated to dryness under reduced pressure.

2.5.1.2. General Procedure for N-Methylation (B).

K₂CO₃ (1.1 equiv.) was added to a stirred solution at ambient temperature of the appropriately substituted cyanamine (1 equiv.) in DMF (5 mL). After 5 minutes methyl iodide was added (2 equiv.) and the mixture was stirred for 18 hours. The solvent was evaporated and the residue was dissolved in water (25 mL). The mixture was extracted with ethyl acetate (3 x 20 mL) and the combined organic fraction was dried over anhydrous MgSO₄, filtrated and evaporated to dryness under reduced pressure.

2.5.1.3. General Procedure for the synthesis of the di- or tri-N-Substituted Guanidines (C).

The appropriately substituted cyanamide (1 mmol) and amine halogen salt (1.1 mmol) in chlorobenzene (200 µL) were dissolved in a screw cap reaction vessel. The reaction vessel was flushed with nitrogen, closed and stirred at 165 °C for 4-18 hours. The reaction mixture was cooled down, dissolved in ethyl acetate (25 mL) and washed with 0.1M HCl (2 x 25 mL) followed by water (25 mL). The pH of the combined aqueous layer was adjusted with K₂CO₃ to pH ≥ 10 and extracted with ethyl acetate (2 x 25 mL). The organic layers were collected and dried over anhydrous MgSO₄, filtrated and evaporated to dryness under reduced pressure. The crude compound was purified by column chromatography. Oily products were converted into their corresponding fumaric or hydrochloric salts by stirring the oil in diethyl ether and drop wise addition of either a saturated solution of fumaric acid in diethyl ether or 2M HCl in diethyl ether. The resulting precipitate was filtered and dried in vacuum.

2.5.1.4. General Procedure for the alkylation of the hydroxyguanidines (D).

The appropriate alkylbromide (3 mmol) was added to a mixture of 3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyphenyl)-1-methylguanidine (14) (1 mmol), potassium carbonate (2 mmol) and potassium iodide (0.1 mmol) in DMF (2 mL). The reaction mixture was heated to 75 °C. After 6 to 24 hours the reaction mixture was cooled to room temperature, diluted with water (25 mL) and washed twice with ethyl acetate (25 mL). The combined organic layer was washed with brine (10 mL). The
organic fraction was collected and dried over anhydrous MgSO₄, filtered and evaporated to dryness under reduced pressure. The crude compound was purified by column chromatography. Oily products were converted into the corresponding fumaric salt by stirring the oil in diethyl ether and drop wise addition of a saturated solution of fumaric acid in diethyl ether. The resulting precipitate was filtered and dried in vacuum.

2.5.1.5. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-methoxyphenyl)-1-methylguanidine (5).

The reaction of \(N\)-(3-methoxyphenyl)-\(N\)-methylcyanamide (25d) (172 mg, 1.06 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (28) (236 mg, 1.12 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et₃N (33/66/1, v/v/v), the title compound as a light yellow oil (114 mg, 0.47 mmol, 45%). \(R_f\) 0.12 (ethyl acetate/petroleum ether (60-80)/Et₃N, 33/66/1, v/v/v). \(^1\)H NMR (CDCl₃) δ 7.35-7.26 (m, 2H, HAryl), 6.94-6.79 (m, 5H, HAryl), 4.01 (bs, 2H, NH), 3.82 (s, 3H, OCH₃), 3.41 (s, 3H, NCH₃), 2.46 (s, 3H, SCH₃). \(^13\)C NMR (CDCl₃) δ 160.52 (Ar-O), 150.83 (Ar-NCH₃), 147.43 (NCN), 145.57 (Ar-NH), 137.63 (Ar-S), 130.33 (Ar), 129.98 (Ar), 124.45 (Ar-Cl), 122.47 (Ar), 121.15 (Ar), 119.00 (Ar), 112.71 (Ar), 112.14 (Ar), 55.35 (OCH₃), 38.71 (NCH₃), 15.91 (SCH₃). The free base was converted into its fumaric acid salt (57 mg, 0.12 mmol, 26%). \(^1\)H NMR (DMSO-\(d_6\)) δ 7.31-7.25 (m, 2H, HAryl), 6.89-6.77 (m, 5H, HAryl), 6.57 (s, 1.6H, fumaric acid), 6.21 (bs, 2H, NH), 3.75 (s, 3H, OCH₃), 3.30 (s, 3H, NCH₃), 2.44 (s, 3H, SCH₃). HRMS calc’d for C₁₆H₁₈ClN₃OS (M+ + H) 336.0932, found 336.0928. HPLC system A: purity >99.9%, tR = 4.35 min.

2.5.1.6. 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-phenylguanidine (9).

The reaction of \(N\)-methyl-\(N\)-phenylcyanamide (30a) (137 mg, 1.04 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (235 mg, 1.12 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et₃N (33/66/1, v/v/v), the title compound as a colorless oil (114 mg, 0.37 mmol, 36%). \(R_f\) 0.14 (ethyl acetate/petroleum ether (60-80)/Et₃N, 33/66/1, v/v/v). \(^1\)H NMR (CDCl₃) δ 7.45-7.24 (m, 6H, HAryl), 6.93 (d, J=2.3Hz, 1H, H Aryl), 6.83 (q, J=8.4Hz, J=2.3Hz, 1H, H Aryl), 3.95 (bs, 2H, NH), 3.43 (s, 3H, NCH₃), 2.47 (s, 3H, SCH₃). \(^13\)C NMR (CDCl₃) δ 150.90 (Ar-NCH₃), 147.46 (NCN), 144.45 (Ar-NH), 137.37 (Ar-S), 130.03 (Ar), 129.74 (Ar), 127.01 (Ar), 126.67 (Ar), 124.54 (Ar-Cl), 122.55 (Ar), 121.24 (Ar), 38.84 (NCH₃), 15.98 (SCH₃). The free base was converted into its fumaric acid salt (132 mg, 0.31 mmol, 84%). \(^1\)H NMR (DMSO-\(d_6\)) δ 7.42-7.18 (m, 6H, HAryl), 6.89-6.84 (m, 2H, HAryl), 6.58 (s, 2.2H, fumaric acid), 6.14 (bs, 2H, NH), 3.31 (s, 3H, NCH₃), 2.44 (s,
2.5.1.7. 1-benzyl-3-(2-chloro-5-(methylthio)phenyl)-1-methylguanidine (10).

The reaction of N-benzyl-N-methylcyanamide (30b) (147 mg, 1.01 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (232 mg, 1.10 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et3N (33/66/1, v/v/v), the title compound as a light tanned solid (236 mg, 0.73 mmol, 73%). Rf 0.12 (ethyl acetate/petroleum ether (60-80)/Et3N, 33/66/1, v/v/v). 1H NMR (CDCl3) δ 7.37-7.28 (m, 6H, H Aryl), 6.90-6.81 (2, 2H, H Aryl), 4.65 (s, 2H, CH2), 4.13 (bs, 2H, NH), 3.01 (s, 3H, NCH3), 2.47 (s, 3H, SCH3). 13C NMR (CDCl3) δ 152.29 (Ar-CH2), 147.71 (NCN), 137.94 (Ar-NH), 137.56 (Ar-S), 129.99 (Ar), 128.60 (Ar), 127.17 (Ar), 127.08 (Ar), 124.70 (Ar-Cl), 122.80 (Ar), 120.88 (Ar), 52.97 (CH2), 35.45 (NCH3), 15.88 (SCH3). HRMS calcd for C16H18ClN3S (M+ + H) 320.0983, found 320.0986. HPLC system A: purity >99.9%, tR = 4.47 min.

2.5.1.8. 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-phenethylguanidine (11).

The reaction of N-methyl-N-phenethylcyanamide (30c) (160 mg, 1.00 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (232 mg, 1.12 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et3N (50/50/1, v/v/v), the title compound as a light green solid (183 mg, 0.55 mmol, 55%). Rf 0.15 (ethyl acetate/petroleum ether (60-80)/Et3N, 50/50/1, v/v/v). 1H NMR (CDCl3) δ 7.37-7.22 (m, 6H, H Aryl), 6.83-6.78 (m, 2H, H Aryl), 3.71 (bs, 2H, NH), 3.63 (t, J=6.9Hz, 2H, CH2-CH2-N), 2.98 (t, J=6.9Hz, 2H, CH2-CH2-N), 2.95 (s, 3H, NCH3), 2.46 (s, 3H, SCH3). 13C NMR (CDCl3) δ 151.79 (Ar-CH2), 147.87 (NCN), 139.62 (Ar-NH), 137.53 (Ar-S), 130.02 (Ar), 129.03 (Ar), 128.63 (Ar), 126.40 (Ar), 124.83 (Ar-Cl), 122.78 (Ar), 120.86 (Ar), 52.07 (CH2CH2NH), 35.86 (NCH3), 34.15 (CH2CH2NH), 15.99 (SCH3). HRMS calcd for C17H20ClN3S (M+ + H) 334.1139, found 334.1140. HPLC system A: purity >99.9%, tR = 4.80 min.

2.5.1.9. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-methoxybenzyl)-1-methylguanidine (12).

The reaction of N-(3-methoxybenzyl)-N-methylcyanamide (30e) (179 mg, 1.02 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (237 mg, 1.13 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et3N (33/66/1, v/v/v), the title compound as a colorless oil (161 mg, 0.46 mmol, 45%). Rf 0.14 (ethyl acetate/petroleum ether (60-80)/Et3N, 33/66/1, v/v/v). 1H NMR (CDCl3) δ 7.35-7.26 (m, 2H, H Aryl), 6.94-6.79 (m, 5H, H Aryl), 4.01 (bs, 2H, NH), 3.82 (s, 3H, OCH3), 3.41 (s, 3H, NCH3), 2.46 (s, 3H, SCH3). 13C NMR (CDCl3) δ 160.15 (Ar-O), 152.38 (Ar-CH2), 147.72 (NCN), 139.78 (Ar-NH),
137.73 (Ar-S), 130.13 (Ar), 129.81 (Ar), 124.80 (Ar-Cl), 122.97 (Ar), 121.14 (Ar), 119.47 (Ar), 112.93 (Ar), 112.64 (Ar), 55.36 (OCH₃), 53.16 (CH₂), 35.74 (NCH₃), 16.04 (SCH₃). The free base was converted into its hydrochloric acid salt (131 mg, 0.34 mmol, 74%). ¹H NMR (DMSO-d₆) δ 9.91 (bs, 1H, NH), 7.82 (bs, 2H, NH₂⁺), 7.55-7.52 (m, 3H, H Aryl), 7.38-7.27 (m, 3H, H Aryl), 4.74 (s, 2H, CH₂), 3.78 (s, 3H, OCH₃), 3.07 (s, 3H, NCH₃). HRMS calcd for C₁₇H₂₀ClN₃OS (M⁺ + H) 350.1088, found 350.1083. HPLC system A: purity >99.9%, tR = 4.47 min.

2.5.1.10.3-(2-chloro-5-(methylthio)phenyl)-1-(3-methoxyphenethyl)-1-methylguanidine (13).

The reaction of N-(3-methoxyphenethyl)-N-methylcyanamide (30f) (189 mg, 0.99 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (232 mg, 1.10 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et₃N (33/66/1, v/v/v), the title compound as a white solid (174 mg, 0.48 mmol, 48%). Rᵣ 0.11 (ethyl acetate/petroleum ether (60-80)/Et₃N, 33/66/1, v/v/v). ¹H NMR (CDCl₃) δ 7.26-7.20 (m, 2H, H Aryl), 6.88-6.76 (m, 5H, H Aryl), 3.81 (s, 3H, OCH₃), 3.68 (bs, 2H, NH), 3.62 (t, J=6.9Hz, 2H, CH₂-CH₂-N), 3.82 (t, J=6.9Hz, 2H, CH₂-CH₂-N), 2.96 (s, 3H, NCH₃), 2.44 (s, 3H, SCH₃). ¹³C NMR (CDCl₃) δ 159.88 (Ar-O), 151.88 (Ar-CH₂), 147.84 (NCN), 141.28 (Ar-NH), 137.59 (Ar-S), 130.07 (Ar), 129.69 (Ar), 124.89 (Ar-Cl), 122.85 (Ar), 121.36 (Ar), 120.96 (Ar), 114.85 (Ar), 111.85 (Ar), 55.26 (OCH₃), 52.08 (CH₂CH₂NH), 35.94 (NCH₃), 34.28 (CH₂CH₂NH), 16.03 (SCH₃). HRMS calcd for C₁₈H₂₂ClN₃OS (M⁺ + H) 364.1245, found 364.1283. HPLC system A: purity >99.9%, tR = 4.78 min.

2.5.1.11.3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyphenyl)-1-methylguanidine (14).

The reaction of N-(3-hydroxyphenyl)-N-methylcyanamide (30g) (355 mg, 2.40 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (232 mg, 2.71 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et₃N (50/50/1, v/v/v), the title compound as a white solid (600 mg, 1.86 mmol, 78%). Rᵣ 0.16 (ethyl acetate/petroleum ether (60-80)/Et₃N, 50/50/1, v/v/v). ¹H NMR (CDCl₃) δ 7.51 (s, 1H, OH), 7.31-7.20 (m, 2H, H Aryl), 6.95 (d, 1H, H Aryl), 6.88-6.78 (m, 4H, H Aryl), 5.21 (bs, 2H, NH), 3.42 (s, 3H, NCH₃), 2.48 (s, 3H, SCH₃). ¹³C NMR (CDCl₃) δ 158.95 (Ar-O), 153.50 (Ar-NCH₃), 145.39 (NCN), 144.33 (Ar-NH), 138.34 (Ar-S), 130.98 (Ar), 130.36 (Ar), 125.56 (Ar-Cl), 123.70 (Ar), 122.66 (Ar), 117.48 (Ar), 115.55 (Ar), 115.44 (Ar), 39.20 (NCH₃), 15.97 (SCH₃). HRMS calcd for C₁₅H₁₆ClN₃OS (M⁺ + H) 322.0775, found 322.0792. HPLC system A: purity >99.9%, tR = 3.48 min.
2.5.1.12. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(difluoromethoxy)phenyl)-1-methylguanidine (15).

The reaction of $N$-(3-(difluoromethoxy)phenyl)-$N$-methylcyanamide (30h) (207 mg, 1.04 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (242 mg, 1.15 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et$_3$N (33/66/1, v/v/v), the title compound as a light yellow oil (182 mg, 0.49 mmol, 47%). $R_f$ 0.09-0.33 (ethyl acetate/petroleum ether (60-80)/Et$_3$N, 33/66/1, v/v/v). $^1$H NMR (CDCl$_3$) $\delta$ 7.42-6.81 (m, 7H, H Aryl), 6.56 (t, $J_{HF}$=73.49Hz, 1H, CHF$_2$), 3.96 (bs, 2H, NH), 3.42 (s, 3H, NCH$_3$), 2.46 (s, 3H, SCH$_3$). $^{13}$C NMR (CDCl$_3$) $\delta$ 151.79 (Ar-O, $J_{CF}$=2.86Hz), 150.64 (Ar-NCH$_3$), 147.15 (NCN), 146.10 (Ar-NH), 137.84 (Ar), 130.10 (Ar), 124.28 (Ar-Cl), 123.38 (Ar), 122.33 (Ar), 121.41 (Ar), 117.89 (Ar), 117.77 (t, $J_{CF}$=260.96Hz, CHF$_2$), 117.02 (Ar), 38.81 (NCH$_3$), 15.92 (SCH$_3$). $^{19}$F NMR (CDCl$_3$) $\delta$: -81.06 (d, $J_{HF}$=73.59Hz, 2F, CHF$_2$). The free base was converted into its fumaric acid salt (174 mg, 0.36 mmol, 80%). $^1$H NMR (DMSO-d$_6$) $\delta$ 7.52-7.13 (m, 4H, H Aryl), 7.22 (t, $J_{HF}$=74.04Hz, 1H, CHF$_2$), 6.96-6.93 (m, 1H, H Aryl), 6.86-6.79 (m, 2H, H Aryl), 6.59 (s, 2H, fumaric acid), 5.98 (bs, 2H, NH), 3.30 (s, 3H, NCH$_3$), 2.43 (s, 3H, SCH$_3$). HRMS calcd for C$_{16}$H$_{16}$ClF$_2$N$_3$OS (M$^+$ + H) 372.0743, found 372.0746. HPLC system A: purity 99.1%, $t_R$ = 4.27 min.

2.5.1.13. 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-(3-(trifluoromethoxy)phenyl)guanidine (16).

The reaction of $N$-methyl-$N$-(3-(trifluoromethoxy)phenyl)cyanamide (30i) (222 mg, 1.03 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (236 mg, 1.12 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et$_3$N (16/83/1, v/v/v), the title compound as a white solid (213 mg, 0.55 mmol, 53%). $R_f$ 0.16-0.28 (ethyl acetate/petroleum ether (60-80)/Et$_3$N, 25/75/1, v/v/v). $^1$H NMR (CDCl$_3$) $\delta$ 7.47-7.37 (m, 1H, H Aryl), 7.32-7.23 (m, 3H, HAryl), 7.14-7.10 (m, 1H, H Aryl), 6.93-6.83 (m, 2H, H Aryl), 3.94 (bs, 2H, NH), 3.44 (s, 3H, NCH$_3$), 2.48 (s, 3H, SCH$_3$). $^{13}$C NMR (CDCl$_3$) $\delta$ 150.59 (Ar-NCH$_3$), 149.92 (Ar-O, $J_{CF}$=1.93Hz), 147.08 (NCN), 146.21 (Ar-NH), 137.95 (Ar-S), 130.69 (Ar), 130.19 (Ar), 124.59 (q, $J_{CF}$=257.47Hz, OCF$_3$), 124.30 (Ar-Cl), 122.36 (Ar), 121.51 (Ar), 119.16 (Ar), 118.36 (Ar), 38.91 (NCH$_3$), 15.92 (SCH$_3$). $^{19}$F NMR (CDCl$_3$) $\delta$ -57.77 (s, 3F, CF$_3$). HRMS calcd for C$_{16}$H$_{16}$ClF$_3$N$_3$OS (M$^+$ + H) 390.0649, found 390.0652. HPLC system A: purity 99.5%, $t_R$ = 5.07 min.

2.5.1.14. 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-(3-(1,1,2,2-tetrafluoroethoxy)phenyl)guanidine (17).

The reaction of $N$-methyl-$N$-(3-(1,1,2,2-tetrafluoroethoxy)phenyl)cyanamide (30j) (248 mg, 1.00 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (231
mg, 1.10 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80) (50/50, v/v), the title compound as a light yellow oil (193 mg, 0.46 mmol, 46%). Rf 0.17 (ethyl acetate/petroleum ether (60-80), 50/50, v/v). \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 7.42 (t, 1H, H\_Aryl) J=8.08Hz, 7.30-7.21 (m, 3H, H\_Aryl), 7.13-7.10 (m, 1H, H\_Aryl), 6.91 (d, J=2.23Hz, 1H, H\_Aryl), 6.84 (dd, J=8.34Hz, J=2.28Hz, 1H, H\_Aryl), 5.94 (tt, J\_HF=53.05Hz, J=2.78Hz, 1H, CHF\(_2\)), 3.92 (bs, 2H, NH), 3.43 (s, 3H, NCH\(_3\)), 2.47 (s, 3H, SCH\(_3\)). \( ^13C \) NMR (CDCl\(_3\)) \( \delta \) 150.60 (Ar-NCH\(_3\)), 149.55 (t, Ar-O, J CF=1.66Hz), 147.20 (NCN), 146.06 (Ar-N), 137.92 (Ar-S), 130.19 (Ar), 124.58 (Ar), 124.35 (Ar-Cl), 122.40 (Ar), 119.94 (Ar), 119.23 (Ar), 116.54 (m, CF\(_2\)CHF\(_2\)), 107.70 (m, CF\(_2\)CHF\(_2\)), 38.90 (NCH\(_3\)), 16.01 (SCH\(_3\)). \( ^19F \) NMR (CDCl\(_3\)) \( \delta \) -88.09 (m, 2F), -136.68 (dt, J\_HF=53.03Hz, J=5.47Hz, 2F). The free base was converted into its fumaric acid salt (135 mg, 0.22 mmol, 47%). \( ^1H \) NMR (DMSO-d\(_6\)) \( \delta \) 7.44 (t, J=8.14Hz, 1H, H\_Aryl), 7.32-7.25 (m, 3H, HAryl), 7.06-6.99 (m, 2H, H Aryl), 6.79 (tt, J\_HF=51.93Hz, J=3.14Hz, 1H, CHF\(_2\)), 6.61 (s, 3.1H, fumaric acid), 6.17 (bs, 2H, NH), 3.32 (s, 3H, NCH\(_3\)), 2.45 (t, 3H, CH\(_3\)). HRMS calcd for C\(_{17}\)H\(_{16}\)ClF\(_4\)N\(_3\)OS (M+ + H) 422.0711, found 422.0704. HPLC system A: purity 99.6%, tR = 4.43 min.

2.5.1.15.1-(2-chloro-5-(methylthio)phenyl)-3-phenylguanidine (18).

The reaction of N-phenylcyanamide (29a) (121 mg, 1.02 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (237 mg, 1.13 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et\(_3\)N (50/50/1, v/v/v), the title compound as a light yellow oil (218 mg, 0.75 mmol, 73%). Rf 0.21 (ethyl acetate). \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 7.33-7.20 (m, 5H, H Aryl), 7.11-7.04 (m, 2H, H Aryl), 6.85 (dd, J=8.38Hz, J=2.31Hz, 1H, H Aryl), 5.32 (bs, 3H, NH), 2.43 (t, 3H, CH\(_3\)). \( ^13C \) NMR (CDCl\(_3\)) \( \delta \) 149.76 (Ar-NH), 144.77 (NCN), 140.26 (Ar-NH), 138.03 (Ar-S), 130.09 (Ar), 129.38 (Ar), 124.35 (Ar-Cl), 124.02 (Ar), 122.73 (Ar), 122.33 (Ar), 121.71 (Ar), 15.90 (SCH\(_3\)). The free base was converted into its fumaric acid salt (189 mg, 0.31 mmol, 84%). \( ^1H \) NMR (DMSO-d\(_6\)) \( \delta \) 7.48 (d, J=7.75Hz, 2H, H Aryl), 7.35-7.23 (m, 3H, HAryl), 7.00-6.88 (m, 3H, H Aryl), 6.03 (bs, 3H, NH), 2.45 (t, 3H, CH\(_3\)). HRMS calcd for C\(_{17}\)H\(_{16}\)ClF\(_4\)N\(_3\) (M+ + H) 292.0670, found 292.0669. HPLC system A: purity >99.9%, tR = 3.80 min.

2.5.1.16.1-benzyl-3-(2-chloro-5-(methylthio)phenyl)guanidine (19).

The reaction of N-benzylcyanamide (29b) (140 mg, 1.06 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (237 mg, 1.13 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et\(_3\)N (50/50/1, v/v/v), the title compound as a white solid (218 mg, 0.71 mmol, 67%). Rf 0.65 (ethyl acetate/petroleum ether (60-80), 50/50, v/v). \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 7.31-7.23 (m, 6H, H Aryl), 6.84-6.78 (m, 2H, H Aryl), 4.67 (bs, 3H, NH), 4.37 (s, 2H, CH\(_2\)).
2.5.1.17.1-(2-chloro-5-(methylthio)phenyl)-3-phenethylguanidine (20).

The reaction of \(N\)-phenethylcyanamide (29c) (153 mg, 1.05 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (231 mg, 1.12 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/Et\(_3\)N (99/1, v/v), the title compound as a light yellow solid (108 mg, 0.34 mmol, 32%). \(R_f\) 0.15 (ethyl acetate/Et\(_3\)N, 99/1, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.32-7.18 (m, 6H, H Aryl), 6.87-6.81 (m, 2H, HAryl), 4.69 (bs, 3H, NH), 3.48 (t, \(J=6.95\)Hz, 2H, N\(\text{CH}_2\)CH\(_2\)), 2.88 (t, \(J=6.93\)Hz, 2H, N\(\text{CH}_2\)CH\(_2\)), 2.43 (s, 3H, CH\(_3\)); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 152.09 (Ar-\(\text{CH}_2\)), 145.85 (NCN), 139.10 (Ar-NH), 137.90 (Ar-S), 130.13 (Ar), 128.88 (Ar), 128.59 (Ar), 126.43 (Ar), 125.34 (Ar-Cl), 123.10 (Ar), 121.71 (Ar), 43.04 (CH\(_2\)CH\(_2\)NH), 35.77 (CH\(_2\)CH\(_2\)NH), 15.89 (SCH\(_3\)).

HRMS calc'd for C\(_{16}\)H\(_{18}\)ClN\(_3\)S (M\(^+\) + H) 320.0983, found 320.0986. HPLC system A: purity >99.9%, \(t_R = 4.22\) min.

2.5.1.18.1-(2-chloro-5-(methylthio)phenyl)-3-(3-methoxyphenyl)guanidine (21).

The reaction of \(N\)-(3-methoxyphenyl)cyanamide (29d) (154 mg, 1.04 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (241 mg, 1.15 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether (60-80)/Et\(_3\)N (50/50/1, v/v/v), the title compound as a colorless oil (233 mg, 0.73 mmol, 70%). \(R_f\) 0.20 (ethyl acetate/petroleum ether (60-80)/Et\(_3\)N, 50/50/1, v/v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.27 (d, \(J=8.40\)Hz, 1H, HAryl), 7.19 (t, \(J=8.06\)Hz, 1H, HAryl), 7.10 (d, \(J=2.29\)Hz, 1H, HAryl), 6.85 (dd, \(J=8.41\)Hz, J=2.34Hz, 1H, HAryl), 6.78 (m, 2H, HAryl), 6.62 (dd, \(J=8.35\)Hz, J=2.38Hz, 1H, HAryl), 5.38 (bs, 3H, NH), 3.73 (s, OCH\(_3\)), 2.43 (s, SCH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 160.56 (Ar-O), 149.66 (Ar-NH), 144.37 (NCN), 141.80 (Ar-NH), 138.14 (Ar-S), 130.13 (Ar), 130.12 (Ar), 124.34 (Ar-Cl), 122.29 (Ar), 121.82 (Ar), 114.77 (Ar), 109.88 (Ar), 108.26 (Ar), 55.33 (OCH\(_3\)), 15.93 (SCH\(_3\)). The free base was converted into its fumaric acid salt (269 mg, 0.12 mmol, 26%). \(^1\)H NMR (DMSO-d\(_6\)) \(\delta\) 7.35 (d, \(J=8.37\)Hz, 1H, HAryl), 7.23-7.14 (m, 2H, HAryl), 7.01-6.91 (m, 3H, HAryl), 6.59 (d, \(J=2.10\)Hz, 1H, HAryl), 6.55 (1.5H, fumaric acid), 6.25 (bs, 3H, NH), 3.72 (s, 3H, N\(\text{CH}_3\)), 2.45 (s, 3H, SCH\(_3\)). HRMS calc'd for C\(_{15}\)H\(_{16}\)ClN\(_3\)OS (M\(^+\) + H) 322.0775, found 322.0772. HPLC system A: purity >99.9%, \(t_R = 3.90\) min.

2.5.1.19.1-(2-chloro-5-(methylthio)phenyl)-3-(3-methoxybenzyl)guanidine (22).

The reaction of \(N\)-(3-methoxybenzyl)cyanamide (29e) (168 mg, 1.04 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (240 mg, 1.14 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/petroleum ether...
(60-80)/Et3N (50/50/1, v/v/v), the title compound as a colorless oil (236 mg, 0.70 mmol, 68%). Rf 0.10 (ethyl acetate/petroleum ether (60-80)/Et3N, 50/50/1, v/v/v).

$^1$H NMR (CDCl$_3$) δ 7.25-7.18 (m, 2H, H$_{Aryl}$), 6.89-6.75 (m, 5H, H$_{Aryl}$), 4.79 (bs, 3H, NH), 4.36 (s, 2H, CH$_2$), 3.77 (s, 3H, OCH$_3$), 2.41 (s, 3H, NCH$_3$).

$^{13}$C NMR (CDCl$_3$) δ 159.90 (Ar-O), 152.37 (Ar-CH$_2$), 145.72 (NCN), 140.37 (Ar-NH), 137.94 (Ar-S), 130.12 (Ar), 129.68 (Ar), 125.25 (Ar-Cl), 123.11 (Ar), 121.79 (Ar), 119.53 (Ar), 113.01 (Ar), 112.73 (Ar), 55.25 (OCH$_3$), 45.59 (CH$_2$), 15.84 (SCH$_3$).

The free base was converted into its hydrochloric acid salt (96 mg, 0.34 mmol, 74%).

$^1$H NMR (DMSO-d$_6$) δ 9.94 (bs, 1H, NH), 8.50 (bs, 1H, NH$_2$), 7.91 (bs, 2H, NH$_2$), 7.55-7.51 (m, 1H, H$_{Aryl}$), 7.34-7.30 (m, 3H, H$_{Aryl}$), 6.96-6.88 (m, 3H, H$_{Aryl}$), 4.49 (d, 2H, CH$_2$, J=5.51Hz), 3.77 (s, 3H, OCH$_3$), 2.50 (s, 3H, SCH$_3$).

HRMS calcd for C$_{16}$H$_{18}$ClN$_3$OS (M$^+$ + H) 336.0932, found 336.0928.

HPLC system A: purity >99.9%, tR = 3.90 min.

2.5.1.20.1. 1-(2-chloro-5-(methylthio)phenyl)-3-(3-methoxyphenethyl)guanidine (23).

The reaction of N-(3-methoxyphenethyl)cyanamide (29f) (182 mg, 1.03 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (240 mg, 1.14 mmol) according to procedure C yielded, after purification over silica with ethyl acetate/Et$_3$N (99/1, v/v), the title compound as a white solid (266 mg, 0.76 mmol, 74%). Rf 0.12 (ethyl acetate/Et$_3$N, 99/1, v/v).

$^1$H NMR (CDCl$_3$) δ 7.28-7.18 (m, 2H, H$_{Aryl}$), 6.84-6.74 (m, 5H, H$_{Aryl}$), 4.27 (bs, 3H, NH), 3.78 (s, 3H, OCH$_3$), 3.48 (t, J=7.81Hz, 2H, NCH$_2$CH$_2$), 2.86 (t, J=7.81Hz, 2H, NCH$_2$CH$_2$), 2.44 (s, 3H, SCH$_3$).

$^{13}$C NMR (CDCl$_3$) δ 159.72 (Ar-O), 152.72 (Ar-CH$_2$), 146.92 (NCl), 140.85 (Ar-NH), 137.71 (Ar-S), 130.05 (Ar), 129.46 (Ar), 125.05 (Ar-Cl), 122.78 (Ar), 121.26 (Ar), 121.22 (Ar), 114.61 (Ar), 111.79 (Ar), 113.05 (Ar), 112.73 (Ar), 55.25 (OCH$_3$), 42.87 (CH$_2$CH$_2$NH), 15.88 (SCH$_3$). HRMS calcd for C$_{17}$H$_{20}$ClN$_3$OS (M$^+$ + H) 350.1088, found 350.1083. HPLC system A: purity >99.9%, tR = 4.18 min.

2.5.1.21.1. 1-(2-chloro-5-(methylthio)phenyl)-3-(3-(difluoromethoxy)phenyl)guanidine (24).

The reaction of N-(3-(difluoromethoxy)phenyl)-N-cyanamide (29h) (184 mg, 1.00 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (231 mg, 1.10 mmol) according to procedure C yielded, after purification over silica with dichloromethane/methanol (95/5, v/v), the title compound as a light yellow oil, which crystallized from dichloromethane as white crystals (200 mg, 0.56 mmol, 56%). Rf 0.33 (dichloromethane/methanol, 95/5, v/v).

$^1$H NMR (CDCl$_3$) δ 7.29-7.20 (m, 2H, H$_{Aryl}$), 7.11 (d, J=2.27Hz, 1H, Ar), 7.02-7.00 (m, 1H, Ar), 6.86 (dd, J=8.41, J=2.29Hz, 1H, Ar), 6.45 (t, J$_{HF}$=74.08Hz, 1H, CHF$_2$), 5.42 (bs, 3H, NH), 2.43 (s, 3H, CH$_3$).

$^{13}$C NMR (CDCl$_3$) δ 152.60 (t, Ar-CH$_2$), 149.42 (Ar-NH), 143.04 (NCN), 143.02 (Ar-NH), 138.37 (Ar-S), 130.43 (Ar), 130.15 (Ar), 124.04 (Ar-Cl), 122.12 (Ar), 122.06 (Ar), 118.86 (Ar),
115.99 (CHF₂, J_CF coupling was obscured by its low intensity), 114.00 (Ar), 113.06 (Ar), 15.83 (SCH₃); ¹⁹F NMR (CDCl₃) δ: -80.80 (d, J_HF=73.95Hz, 2F, CHF₂). HRMS calcld for C₁₅H₁₄ClF₂N₃OS (M+ + H) 358.0576, found 358.0576. HPLC system A: purity >99.9%, t_R = 3.93 min.

2.5.1.22.1-(2-chloro-5-(methylthio)phenyl)-3-(3-(trifluoromethoxy)phenyl)guanidine (25).

The reaction of N-(3-(trifluoromethoxy)phenyl)-N-cyanamide (29i) (202 mg, 1.00 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (33) (231 mg, 1.10 mmol) according to procedure C yielded, after purification over silica with dichloromethane/methanol (95/5, v/v), the title compound as a light yellow solid (93 mg, 0.25 mmol, 25%). R_f 0.29 (dichloromethane/methanol, 95/5, v/v). ¹H NMR (CDCl₃) δ 7.40 (d, J=8.46Hz, 1H, H_Aryl), 7.28 (dt, J=7.91, J=0.58Hz, 1H, H_Aryl), 7.26 (d, J=2.56 Hz, 1H, H_Aryl), 7.15 (dd, J=8.46, J=2.34Hz, 1H, H_Aryl), 6.93-6.81 (m, 3H, HAryl), 5.22 (bs, 3H, NH), 3.30 (s, 3H, NCH₃), 2.51 (s, 3H, SCH₃); ¹³C NMR (CDCl₃) δ 149.96 (q, Ar-O, J_CF=1.77Hz), 149.16, 143.69 (CN), 142.70 (Ar-NH), 138.40 (Ar-S), 131.01 (Ar), 130.13 (Ar), 123.82 (Ar-Cl), 122.14 (Ar), 121.90 (Ar), 120.26 (Ar), 115.39 (Ar), 114.65 (Ar), 120.52 (q, J_CF=257.26Hz, OCF₃), 15.86 (SCH₃); ¹⁹F NMR (CDCl₃) δ: -57.53 (s, 3F, CF₃). HRMS calcld for C₁₅H₁₃ClF₃N₃OS (M+ + H) 376.0476, found 376.0476; HPLC system A: purity 99.9%, t_R = 4.52 min.

2.5.1.23.1-(2-chloro-5-(methylthio)phenyl)-3-(3-(difluoromethoxy)phenyl)-1-methylguanidine (26).

The reaction of N-(3-(difluoromethoxy)phenyl)-N-cyanamide (29h) (93 mg, 0.51 mmol) with 2-chloro-N-methyl-5-(methylthio)aniline hydrochloride (34) (123 mg, 0.55 mmol) according to procedure C yielded, after purification over silica with dichloromethane/methanol (95/5, v/v), the title compound as a colorless oil (80 mg, 0.22 mmol, 43%). R_f 0.34 (dichloromethane/methanol, 95/5 v/v). ¹H NMR (CDCl₃) δ 7.39 (d, J=8.45Hz, 1H, H_Aryl), 7.38-7.22 (m, 2H, H_Aryl), 7.15 (dd, J=8.46Hz, J=2.29Hz, 1H, HAryl), 6.83-6.71 (m, 3H, H_Aryl), 6.51 (t, J_HF=74.60Hz, 1H, CHF₂), 3.85 (bs, 2H, NH), 3.29 (s, 3H, NCH₃), 2.50 (s, 3H, SCH₃); ¹³C NMR (CDCl₃) δ 152.50 (Ar-O, J_CF=2.79Hz), 152.00 (Ar-NH), 143.69 (CN), 141.64 (Ar-NCH₃), 139.56 (Ar-S), 131.01 (Ar), 130.23 (Ar-Cl), 127.90 (Ar), 126.80 (Ar), 120.39 (Ar), 116.21 (CHF₂, J_CF coupling not observed), 114.25 (Ar), 112.69 (Ar), 37.41 (NCH₃), 15.77 (SCH₃); ¹⁹F NMR (CDCl₃) δ: -80.24 (d, J_HF=74.32, 2F, CHF₂). The free base was converted into its fumaric acid salt (43 mg, 0.09 mmol, 42%). ¹H NMR (DMSO-d₆) δ 7.48-7.19 (m, 4H, H_Aryl), 7.17 (t, J_HF=74.64Hz, 1H, CHF₂), 6.88-6.67 (m, 3H, H_Aryl), 6.55 (s, 1.6H, fumaric acid), 6.01 (bs, 2H, NH), 3.32 (s, 3H, NCH₃), 2.50 (s, 3H, SCH₃). HRMS calcld for C₁₆H₁₆ClF₂ClN₃OS (M+ + H) 372.0733, found 372.0733. HPLC system A: purity 97.9%, t_R = 4.40 min.
2.5.1.24. 1-(2-chloro-5-(methylthio)phenyl)-3-(3-(trifluoromethoxy)phenyl)-1-methylguanidine (27).

The reaction of N-(3-(trifluoromethoxy)phenyl)-N-cyanamide (29i) (101 mg, 0.50 mmol) with 2-chloro-N-methyl-5-(methylthio)aniline hydrochloride (34) (123 mg, 0.55 mmol) according to procedure C yielded, after purification over silica with dichloromethane/methanol (95/5, v/v), the title compound as a colorless oil (114 mg, 0.29 mmol, 59%). $R_f$ 0.29 (dichloromethane/methanol, 95/5, v/v). $^1$H NMR (CDCl$_3$) δ 7.40 (d, $J=8.46$ Hz, 1H, H Aryl), 7.28 (dt, $J=7.91$, $J=0.58$ Hz, 1H, H Aryl), 7.26 (d, $J=2.56$ Hz, 1H, H Aryl), 7.15 (dd, $J=8.46$, $J=2.34$ Hz, 1H, H Aryl), 6.93-6.81 (m, 3H, H Aryl), 3.80 (bs, 2H, NH), 3.30 (s, 3H, NCH$_3$), 2.51 (s, 3H, SCH$_3$); $^{13}$C NMR (CDCl$_3$) δ 151.80 (Ar-NH), 150.69 (NCN), 150.24 (Ar-O, $J_{CF}=1.77$ Hz), 141.56 (Ar-NCH$_3$), 139.62 (Ar-S), 131.03 (Ar), 130.25 (Ar), 130.21 (Ar-Cl), 127.90 (Ar), 126.85 (Ar), 121.84 (Ar), 120.62 (q, $J_{CF}=256.70$ Hz, CF$_3$), 116.02 (Ar), 114.26 (Ar), 37.45 (NCH$_3$), 15.76 (SCH$_3$); $^{19}$F NMR (CDCl$_3$) δ: -57.53 (s, 3F, CF$_3$). HRMS calcd for C$_{16}$H$_{15}$ClF$_3$N$_3$OS (M$^+$ + H) 390.0649, found 390.0641; HPLC system A: purity 97.4%, tR = 5.08 min.

2.5.1.25. N-phenylcyanamide (29a).

The reaction of aniline (28a) (759 mg, 8.15 mmol) according to procedure A yielded the title compound as a yellow oil (276 mg, 3.81 mmol, 47%); $R_f$ 0.83 (ethyl acetate/petroleum ether (60-80), 50/50, v/v). $^1$H NMR (CDCl$_3$) δ 7.48 (s, 1H, NH), 7.39-7.32 (m, 2H, H Aryl), 7.13-7.06 (m, 3H, H Aryl); $^{13}$C NMR (CDCl$_3$) δ 151.80 (Ar-NH), 129.76 (Ar), 123.62 (Ar), 115.54 (Ar), 112.00 (NCN).


The reaction of benzylamine (28b) (864 mg, 8.06 mmol) according to procedure A yielded the title compound as a white solid (487 mg, 3.69 mmol, 46%); $R_f$ 0.65 (ethyl acetate/petroleum ether (60-80), 50/50, v/v). $^1$H NMR (CDCl$_3$) δ 7.32-7.20 (m, 5H, HAryl), 4.56 (bs, 1H, NH), 4.02 (bs, 1H, NH), 3.34 (q, $J=6.9$ Hz, 2H, CH$_2$); $^{13}$C NMR (CDCl$_3$) δ 37.38 (Ar-N), 128.90 (Ar), 128.38 (Ar), 116.21 (NCN), 47.37 (CH$_2$CH$_2$NH), 35.95 (CH$_2$CH$_2$NH).

2.5.1.27. N-phenethylcyanamide (29c).

The reaction of phenethylamine (28c) (984 mg, 8.12 mmol) according procedure A yielded the title compound as a white solid (535 mg, 3.64 mmol, 45%); $R_f$ 0.70 (ethyl acetate/ petroleum ether (60-80), 50/50, v/v). $^1$H NMR (CDCl$_3$) δ 7.41-7.23 (m, 5H, HAryl), 4.02 (bs, 1H, NH), 3.34 (q, $J=6.9$ Hz, 2H, CH$_2$CH$_2$NH), 2.94 (t, $J=7.1$ Hz, 2H, CH$_2$CH$_2$NH); $^{13}$C NMR (CDCl$_3$) δ 37.38 (Ar-N), 128.88 (Ar), 128.86 (Ar), 126.99 (Ar), 116.21 (NCN), 47.37 (CH$_2$CH$_2$NH), 35.95 (CH$_2$CH$_2$NH).
2.5.1.28. N-(3-methoxyphenyl)cyanamide (29d).
The reaction of 3-methoxyaniline (28d) (991 mg, 8.05 mmol) according to the
procedure A yielded the title compound as a white solid (575 mg, 3.88 mmol, 48%); Rf
0.71 (ethyl acetate/petroleum ether (60-80), 50/50, v/v). 1H NMR (CDCl3) δ 7.45 (s,
1H, NH), 7.38-7.32 (m, 1H, H_Aryl), 6.78-6.71 (m, 3H, H_Aryl), 3.92 (s, 3H, CH3). 13C NMR
(CDCl3) δ 160.83 (Ar-O), 138.68 (Ar-N), 130.69 (Ar), 111.78 (NCN), 109.20 (Ar),
107.97 (Ar), 101.67 (Ar), 55.46 (CH3).

2.5.1.29. N-(3-methoxybenzyl)cyanamide (29e).
The reaction of 3-methoxybenzylamine (28e) (998 mg, 7.28 mmol) according to
procedure A yielded the title compound as a white solid (465 mg, 2.87 mmol, 39%);
Rf 0.43 (dichloromethane/methanol, 95/5, v/v). 1H NMR (CDCl3) δ 7.20-7.14 (m, 1H,
HAryl), 6.79-6.73 (m, 3H, HAryl), 4.83 (bs, 1H, NH), 3.99 (d, J=5.7Hz, 2H, CH2), 3.68 (s, 3H,
CH3). 13C NMR (CDCl3) δ 159.81 (Ar-O), 137.98 (Ar-N), 129.87 (Ar), 119.91 (Ar) 116.62
(NCN), 113.69 (Ar), 113.24 (Ar), 55.20 (CH3), 49.64 (CH2). MS m/z 163.1 (M+ + H).

2.5.1.30. N-(3-methoxyphenethyl)cyanamide (29f).
The reaction of 3-methoxyphenethylamine (28f) (1212 mg, 8.02 mmol) according to
procedure A yielded the title compound as a white solid (636 mg, 3.61 mmol, 45%);
Rf 0.49 (dichloromethane/methanol, 95/5, v/v). 1H NMR (CDCl3) δ 7.25 (t, J=7.9Hz, 1H,
HAryl), 6.82-6.76 (m, 3H, H Aryl), 4.15 (bs, 1H, NH), 3.80 (s, 3H, CH3), 3.30 (q, J=13.2Hz,
J=6.5Hz, 2H, CH2CH2NH), 2.88 (t, J=7.0Hz, 2H, CH2CH2NH). 13C NMR (CDCl3) δ 159.90
(Ar-O), 138.98 (Ar-N), 129.85 (Ar), 121.14 (Ar), 116.28 (NCN), 114.65 (Ar), 112.24
(Ar), 55.24 (CH3), 47.22 (CH2CH2NH), 35.95 (CH2CH2NH). MS m/z 177.5 (M+ + H).

2.5.1.31. N-(3-hydroxyphenyl)cyanamide (29g).
The reaction of 3-aminophenol (28g) (10.0 g, 91.63 mmol) according to procedure A
yielded the title compound as a white solid (5.94 g, 44.25 mmol, 48%); Rf 0.39 (ethyl
acetate/petroleum ether (60-80), 33/67, v/v). 1H NMR (DMSO-d6) δ 9.98 (s, 1H, OH),
9.60 (bs, 1H, NH), 7.14-7.07 (m, 1H, H_Aryl), 6.44-6.36 (m, 3H, H_Aryl). 13C NMR (DMSO-d6)
δ 158.59 (Ar-O), 139.74 (Ar-N), 130.59 (Ar), 112.10 (NCN), 109.83 (Ar), 105.65 (Ar),
101.99 (Ar).

2.5.1.32. N-(3-(difluoromethoxy)phenyl)cyanamide (29h).
The reaction of 3-(difluoromethoxy)aniline (28h) (1.60 g, 10.0 mmol) according to
procedure A yielded, after purification over silica with ethyl acetate/petroleum ether
(60-80) (25/75, v/v), the title compound as a light brown oil which solidified upon
standing (683 mg, 3.71 mmol, 37%); Rf 0.24 (ethyl acetate/petroleum ether (60-80),
25/75, v/v). 1H NMR (CDCl3) δ 7.31-7.22 (m, 2H, H_Aryl), 6.87-6.76 (m, 2H, H_Aryl), 6.77 (s,
1H, NH), 6.48 (t, J_HF=73.5Hz, 1H, CHF2). 13C NMR (CDCl3) δ 152.30 (Ar-O), 138.96 (Ar-
2.5.1.33. \(N\)-(3-(trifluoromethoxy)phenyl)cyanamide (29i).

The reaction of 3-(trifluoromethoxy)aniline (28i) (1.780 g, 10.05 mmol) according to procedure A yielded, after purification over silica with ethyl acetate/petroleum ether (60-80) (25/75, v/v), the title compound as a white solid (583 mg, 2.88 mmol, 29%); \(R_f\) 0.43 (ethyl acetate/petroleum ether (60-80), 25/75, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.39-7.32 (m, 2H, HAryl), 6.99-6.93 (m, 2H, HAryl), 6.88 (s, 1H, NH). \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 150.34 (Ar-O), 139.03 (Ar-N), 131.15 (Ar), 120.47 (q, CF\(_3\), \(J_{CF}=258.08\) Hz), 115.84 (Ar), 113.82 (Ar), 111.06 (NCN), 108.58 (Ar).

2.5.1.34. \(N\)-(3-(1,1,2,2-tetrafluoroethoxy)phenyl)cyanamide (29j).

The reaction of 3-(1,1,2,2-tetrafluoroethoxy)aniline (28j) (1.033 g, 4.94 mmol) according to procedure A yielded, after purification over silica with ethyl acetate/petroleum ether (60-80) (20/80, v/v), the title compound as a colorless oil (475 mg, 2.03 mmol, 41%); \(R_f\) 0.22 (ethyl acetate/petroleum ether (60-80), 20/80, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.34 (t, \(J=8.17\)Hz, 1H, HAryl), 6.98-6.89 (m, 3H, HAryl), 5.90 (dt, \(J_{HF}=52.96, 2.85\)Hz, 1H, CHF\(_2\)). \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 149.99 (Ar-O), 138.86 (Ar-N), 131.01 (Ar), 128.84 (Ar-N), 131.11 (NCN), 109.22 (Ar), 107.70 (tt, \(J_{CF}=252.06, J_{CF}=41.24\)Hz, CF\(_2\)CHF\(_2\)), 19F NMR (CDCl\(_3\)) \(\delta\) -88.26 (m, 2F), 136.80 (dt, \(J_{HF}=53.29, J_{CF}=5.57\)Hz, 2F).

2.5.1.35. \(N\)-methyl-\(N\)-phenylcyanamide (30a).

The reaction of \(N\)-phenylcyanamide (29a) (468 mg, 3.96 mmol) according to procedure B yielded the title compound as a yellow oil (476 mg, 3.60 mmol, 91%); \(R_f\) 0.90 (dichloromethane/methanol, 95/5, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.42-7.36 (m, 2H, HAryl); 7.14-7.08 (m, 3H, HAryl); 3.33 (s, 3H, CH\(_3\)). \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 140.38 (Ar-N), 129.62 (Ar), 123.37 (Ar), 114.87 (Ar), 114.14 (NCN), 36.81 (CH\(_3\)).

2.5.1.36. \(N\)-benzyl-\(N\)-methylcyanamide (30b).

The reaction of \(N\)-benzylcyanamide (29b) (487 mg, 3.86 mmol) according to procedure B yielded the title compound as a yellow oil (487 mg, 3.33 mmol, 90%); \(R_f\) 0.91 (dichloromethane/methanol, 95/5, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.34-7.35 (m, 5H, HAryl), 4.18 (s, 2H, CH\(_2\)), 2.80 (s, 3H, CH\(_3\)). \(^13\)C NMR (CDCl\(_3\)) \(\delta\) 134.33 (Ar), 128.84 (Ar), 128.53 (Ar), 128.35 (Ar), 118.76 (NCN), 57.04 (CH\(_2\)), 37.79 (CH\(_3\)).

2.5.1.37. \(N\)-methyl-\(N\)-phenethylcyanamide (30c).

The reaction of \(N\)-phenethylcyanamide (29b) (532 mg, 3.64 mmol) according to procedure B yielded the title compound as a yellow oil (576 mg, 3.60 mmol, 99%); \(R_f\)
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0.91 (dichloromethane/methanol, 95/5, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.37-7.23 (m, 5H, H\(_{Aryl}\)), 3.22 (q, J=6.6Hz, 2H, CH\(_2\)CH\(_2\)N), 2.96 (t, J=7.4Hz, 2H, CH\(_2\)CH\(_2\)N), 2.82 (s, 3H, CH\(_3\)). \(^1^3\)C NMR (CDCl\(_3\)) \(\delta\) 137.51 (Ar-N), 128.70 (Ar), 126.83 (Ar), 118.79 (NCN), 54.32 (CH\(_2\)CH\(_2\)N), 39.17 (CH\(_3\)), 33.83 (CH\(_2\)CH\(_2\)N).

2.5.1.38. \(N\)-(3-methoxyphenyl)-\(N\)-methylcyanamide (30d).
The reaction of \(N\)-(3-methoxyphenyl)cyanamide (29d) (426 mg, 2.88 mmol) according to procedure B yielded the title compound a colorless oil which solidifies upon standing to a pale white solid (445 mg, 2.74 mmol, 95%); Rf 0.83 (dichloromethane/methanol, 95/5, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.27-7.20 (m, 1H, H\(_{Aryl}\)), 6.65-6.59 (m, 3H, H\(_{Aryl}\)), 3.78 (s, 3H, OCH\(_3\)), 3.27 (s, 3H, NCH\(_3\)). \(^1^3\)C NMR (CDCl\(_3\)) \(\delta\) 160.83 (q-Ar-O), 141.78 (q-Ar-N), 130.48 (t-Ar), 114.05 (NCN), 108.67 (t-Ar), 107.21 (t-Ar), 101.46 (t-Ar), 55.45 (OCH\(_3\)), 36.90 (NCH\(_3\)). MS m/z 176.8 (M\(^+\) + H).

2.5.1.39. \(N\)-(3-methoxybenzyl)-\(N\)-methylcyanamide (30e).
The reaction of \(N\)-(3-methoxybenzyl)cyanamide (29e) (297 mg, 1.83 mmol) according to procedure B yielded the title compound as a yellow oil (294 mg, 1.67 mmol, 91%); Rf 0.86 (dichloromethane/methanol, 95/5, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.34-7.28 (m, 1H, H\(_{Aryl}\)), 6.93-6.88 (m, 3H, H\(_{Aryl}\)), 4.13 (s, 2H, CH\(_2\)), 3.83 (s, 3H, OCH\(_3\)), 2.79 (s, 3H, NCH\(_3\)). \(^1^3\)C NMR (CDCl\(_3\)) \(\delta\) 160.12 (Ar-O), 135.97 (Ar-N), 130.06 (Ar), 120.65 (Ar), 118.93 (NCN), 114.15 (Ar), 113.94 (Ar), 57.20 (CH\(_2\)), 55.36 (OCH\(_3\)), 37.90 (NCH\(_3\)). MS m/z 176.8 (M\(^+\) + H).

2.5.1.40. \(N\)-(3-methoxyphenethyl)-\(N\)-methylcyanamide (30f).
The reaction of \(N\)-(3-methoxyphenethyl)cyanamide (29f) (464 mg, 2.63 mmol) according to procedure B yielded the title compound as a yellow oil (481 mg, 2.53 mmol, 96%); Rf 0.89 (dichloromethane/methanol, 95/5, v/v). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.28-7.22 (m, 1H, H\(_{Aryl}\)), 6.84-6.78 (m, 3H, H\(_{Aryl}\)), 3.81 (s, 3H, OCH\(_3\)), 3.22 (q, J=6.4Hz, 2H, CH\(_2\)CH\(_2\)N), 2.92 (t, J=7.0Hz, 2H, CH\(_2\)CH\(_2\)N), 2.83 (s, 3H, NCH\(_3\)). \(^1^3\)C NMR (CDCl\(_3\)) \(\delta\) 159.88 (Ar-O), 139.09 (Ar-N), 129.74 (Ar), 121.01 (Ar), 118.26 (NCN), 114.54 (Ar), 112.16 (Ar), 55.20 (OCH\(_3\)), 54.26 (CH\(_2\)CH\(_2\)N), 39.20 (NCH\(_3\)), 33.90 (CH\(_2\)CH\(_2\)N). MS m/z 191.4 (M\(^+\) + H).

2.5.1.41. \(N\)-(3-hydroxyphenyl)-\(N\)-methylcyanamide (30g).
Methyl iodide (2.7 mL, 43.2 mmol) was added to a suspension of \(N\)-(3-hydroxyphenyl)cyanamide (29g) (5.87 g, 43.8 mmol) and potassium carbonate (6.05 g, 43.8 mmol) in DMF (30 mL). The suspension was refluxed for 45 minutes before cooling down and filtration. The filtrate was evaporated and the residue was dissolved in ethyl acetate (60 mL) and washed twice with water (60 mL). The organic fraction was collected, dried with anhydrous MgSO\(_4\), filtered and evaporated to dryness. The
dark brown liquid was dissolved in a minimum amount of methanol and water was added. The title compound was obtained as light brown crystals (4.21 g, 28.4 mmol, 65%); \( R_f \) 0.42 (ethyl acetate/petroleum ether (60-80), 33:67). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 9.71 (s, 1H, OH), 7.23-7.16 (m, 1H, HAryl), 6.56-6.49 (m, 3H, HAryl), 3.27 (s, 3H, CH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \) 158.56 (Ar-O), 141.65 (Ar-N), 130.52 (Ar), 114.00 (NCN), 110.33 (Ar), 105.33 (Ar), 102.00 (Ar), 36.41 (CH\(_3\)).

2.5.1.42. \( N \)-(3-(difluoromethoxy)phenyl)-\( N \)-methylcyanamide (30h).

The reaction of \( N \)-(3-(difluoromethoxy)phenyl)cyanamide (29h) (683 mg, 3.71 mmol) according to procedure B yielded, after purification over silica with ethyl acetate/petroleum ether (60-80) (1/6, v/v), the title compound as a yellow oil (477 mg, 2.41 mmol, 65%); \( R_f \) 0.48 (ethyl acetate/petroleum ether (60-80), 20/80 v/v). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 7.40-7.34 (t, \( J=8.18 \) Hz, 1H, HAryl), 6.98-6.94 (m, 1H, HAryl), 6.88-6.83 (m, 2H, HAryl), 6.77 (s, 1H, NH), 6.54 (m, \( J_{HF}=73.5 \) Hz, 1H, CHF\(_2\)), 3.34 (s, 3H, NMe). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \) 152.24 (t, Ar-O, \( J_{CF}=2.96 \)Hz), 142.12 (Ar-N), 130.92 (Ar), 115.73 (t, CHF\(_2\), \( J_{CF}=206.59 \)Hz), 113.86 (Ar), 113.40 (NCN), 110.64 (Ar), 36.93 (CH\(_3\)). \(^{19}\)F NMR (CDCl\(_3\)) \( \delta \) -81.11 (d, \( J_{HF}=73.38 \)Hz, 2F, CHF\(_2\)). MS \( m/z \) 199.1 (M\(^+\) + H).

2.5.1.43. \( N \)-methyl-\( N \)-(3-(trifluoromethoxy)phenyl)cyanamide (30i).

The reaction of \( N \)-(3-(trifluoromethoxy)phenyl)cyanamide (29i) (583 mg, 2.88 mmol) according procedure B yielded, after purification over silica with ethyl acetate/petroleum ether (60-80) (20/80, v/v), the title compound as a colorless oil (384 mg, 1.78 mmol, 62%); \( R_f \) 0.49 (ethyl acetate/petroleum ether (60-80), 20/80 v/v). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 7.56 (t, \( J=8.3 \)Hz, 1H, HAryl), 7.22-7.07 (m, 3H, HAryl), 3.51 (s, 3H, CH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \) 150.36 (Ar-O), 142.20 (Ar-N), 130.99 (Ar), 120.47 (q, \( J_{CF}=257.95 \)Hz, OCF\(_3\)), 115.49 (Ar), 114.32 (NCN), 113.35 (Ar), 107.99 (Ar), 37.05 (CH\(_3\)). \(^{19}\)F NMR (CDCl\(_3\)) \( \delta \) -57.78 (s, 3F, CF\(_3\)). MS \( m/z \) 217.0 (M\(^+\) + H).

2.5.1.44. \( N \)-methyl-\( N \)-(3-(1,1,2,2-tetrafluoroethoxy)phenyl)cyanamide (30j).

The reaction of \( N \)-(3-(1,1,2,2-tetrafluoroethoxy)phenyl)cyanamide (29j) (475 mg, 2.03 mmol) according to procedure B yielded, after purification over silica with ethyl acetate/petroleum ether (60-80) (20/80, v/v), the title compound as a yellow oil (454 mg, 1.83 mmol, 90%); \( R_f \) 0.32 (ethyl acetate/petroleum ether (60-80), 20/80, v/v). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 7.38 (t, \( J=8.26 \)Hz, 1H, HAryl), 7.04-6.89 (m, 3H, HAryl), 5.90 (dt, \( J_{HF}=53.05 \), \( J_{HF}=5.03 \)Hz, 1H, CHF\(_2\)), 3.34 (s, 3H, CH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \) 150.00 (Ar-O), 142.07 (Ar-N), 130.83 (Ar), 116.31 (Ar), 116.08 (m, CF\(_2\)CHF\(_2\)), 113.37 (NCN), 113.09 (Ar), 108.62 (Ar), 107.69 (m, CF\(_2\)CHF\(_2\)), 37.03 (CH\(_3\)). \(^{19}\)F NMR (CDCl\(_3\)) \( \delta \) -88.16 (m, 2F), -136.77 (dt, \( J_{HF}=52.76 \), \( J_{HF}=5.52 \)Hz, 2F).
2.5.1.45. 2-chloro-5-(methylthio)aniline hydrochloride (33).

Triethylamine (11 ml, 79 mmol) was added to a stirred solution of 2-chloro-5-(methylthio)benzoic acid (31) (10.1 g, 49.6 mmol) in t-butanol (40 mL). Diphenyl phosphorazidate (12 ml, 55.7 mmol) was added dropwise at a rate of one drop per second. The reaction mixture was slowly heated and refluxed for 6 hours. The reaction mixture was cooled and the solvents were evaporated under reduced pressure. The residue (32, \( ^1H \) NMR (CDCl₃) \( \delta \) 8.14 (d, 1H, Ar, J=2.20Hz), 7.22 (d, 1H, Ar, J=8.44Hz), 6.99 (bs, 1H, NH), 6.84 (dd, 1H, Ar, J=8.44Hz, 2.32Hz), 2.49 (s, 3H, CH₃), 1.54 (s, 9H, t-Bu). \( ^{13}C \) NMR (CDCl₃) \( \delta \) 152.19 (CO), 138.78 (Ar), 135.48 (Ar), 128.96 (Ar), 121.14 (Ar), 118.17 (Ar), 117.03 (Ar), 81.24 (C(CH₃)₃), 15.91 (C(CH₃)₃).) was dissolved in THF (25 mL) and concentrated hydrochloric acid / water (1:1) (25 mL) was added. The reaction mixture was refluxed for 6 hours and cooled to room temperature. The organic solvents were evaporated under reduced pressure and the pH was adjusted with NaOH (25% w/v in water) to 12. The mixture was extracted with ethyl acetate (4 x 50 ml). The organic fractions were combined and washed with water (30 ml). The organic layer was collected and dried with anhydrous MgSO₄, filtered and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (ethyl acetate/petroleum ether (60-80), 1/7, v/v). The fraction containing the product was evaporated and dissolved in diethyl ether, 2M hydrochloric acid in diethyl ether (20 mL) was added to the stirred solution and filtrated. The title compound was obtained as a brown solid (7.5 g, 35.7 mmol, 72%); \( R_f \) 0.38 (ethyl acetate/petroleum ether (60-80), 1/6, v/v). \( ^1H \) NMR (DMSO-d₆) \( \delta \) 7.16 (d, J=8.35Hz, 1H, HAryl), 6.55-6.51 (m, 2H, HAryl), 4.22 (bs, 1H, NH₃⁺, HCl), 2.41 (s, 3H, CH₃); \( ^{13}C \) NMR (DMSO-d₆) \( \delta \) 138.24 (Ar-S), 129.86 (Ar), 119.99 (Ar), 118.30 (Ar-Cl), 116.78 (Ar), 14.82 (CH₃), the aromatic carbon of the aniline (Ar-NH₂) was obscured due to its low intensity.

2.5.1.46. 2-chloro-N-methyl-5-(methylthio)aniline hydrochloride (34).

Under nitrogen atmosphere, n-butyllithium (1.6M in hexanes, 1.68 mL, 2.69 mmol) was slowly added to a stirred solution of 2-chloro-5-(methylthio)aniline (33) (467 mg, 2.69 mmol) in THF (dry, 10 mL) at 0 °C. After 15 minutes methyl iodide (168 µL, 2.69 mmol) was added and the reaction was allowed to warm up to room temperature and stirring was continued for 4 hours. The reaction mixture was evaporated and partitioned between diethyl ether (50 mL) and water (50 mL). The organic layer was collected, dried with anhydrous MgSO₄, filtrated and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (hexanes/chloroform, 2/1, v/v) yielding the free base as a light yellow oil (411 mg, 2.19 mmol, 81%); \( R_f \) 0.76 (hexanes/chloroform 2/1, v/v). \( ^1H \) NMR (CDCl₃) \( \delta \) 7.15 (dd, J=6.74, J=2.05Hz,1H, HAryl), 6.55-6.51 (m, 2H, HAryl), 4.22 (bs, 1H, NH), 2.90 (s, 3H,
NCH₃), 2.47 (s, 3H, SCH₃); ¹³C NMR (CDCl₃) δ 145.07 (Ar-N), 138.21 (Ar-S), 129.13 (Ar), 116.25 (Ar-Cl), 115.12 (Ar), 108.99 (Ar), 30.36 (NCH₃), 16.25 (SCH₃). The free base was converted into its hydrochloric acid salt (448 mg, 2.00 mmol, 91%). ¹H NMR (DMSO-d₆) δ 7.16 (dd, J=8.72, J=1.72Hz, 1H, H_Aryl), 6.49-6.45 (m, 2H, H_Aryl), 5.48 (bs, 1H, NH), 2.75 (s, 3H, NCH₃), 2.44 (s, 3H, SCH₃).

2.5.1.47. t-butyl 2-chloro-5-(methylthio)phenyl(cyano)carbamate (35).
Sodium hydride (60%, 0.56 g, 13.90 mmol) was added to a cooled (ice-water bath) solution of t-butyl (2-chloro-5-(methylthio)phenyl)carbamate (32) (3.74g, 13.66 mmol) in THF (20 mL). After 15 min a solution of cyanic bromide (1.59g, 14.99 mmol) in THF (10 mL) was added via an addition funnel. The reaction mixture was stirred for 15 min before the ice-water bath was removed and stirring was continued for 16 h. The reaction was quenched with water (5 mL) and stirred for 5 min. The reaction mixture was diluted with ethyl acetate (50 mL) and water (100 mL). The organic layer was collected and washed with water (100 mL), collected, dried over anhydrous MgSO₄, filtrated and evaporated to dryness under reduced pressure. The residue was purified over silica with ethyl acetate/petroleum ether (60-80) (10/90, v/v), yielding the title compound as light yellow solid. The product was crystallized from ethyl acetate/hexanes to obtain the title compound as a white solid (2.86g, 9.57 mmol, 70%). Rf 0.53 (ethyl acetate/petroleum ether (60-80), 20/80, v/v). ¹H NMR (CDCl₃) δ 7.42-7.38 (m, 1H, H_Aryl), 7.27-7.22 (m, 2H, H_Aryl), 2.50 (s, 3H, CH₃), 1.56 (s, 9H, t-Bu); ¹³C NMR (CDCl₃) 149.44 (CO), 139.99 (Ar-N), 132.65 (Ar-S), 130.73 (Ar), 128.84 (Ar-Cl), 126.64 (Ar), 108.01 (NCN), 87.07 (C(CH₃)₃), 27.82 (C(CH₃)₃), 15.85 (S-Me).

2.5.1.48. N-(2-chloro-5-(methylthio)phenyl)-2,2,2-trifluoroacetamide (36).
A solution of trifluoroacetic anhydride (340 µL, 2.41 mmol) in dichloromethane (2.5 mL) was added dropwise to a cooled (ice-water bath) solution of 2-chloro-5-(methylthio)aniline (33) (347 mg, 2.00 mmol) and triethylamine (350 µL, 2.51 mmol) in dichloromethane (6 mL). The ice-water bath was removed and stirring was continued for 1 h. Dichloromethane (15 mL) was added and the reaction mixture was washed with brine (25 mL). The organic layer was collected, dried over anhydrous MgSO₄, filtrated and evaporated to dryness under reduced pressure. The residue was purified over silica with ethyl acetate/hexanes (5/95, v/v), yielding the title compound as white crystals (506 mg, 1.88 mmol, 94%). Rf 0.37 (ethyl acetate/hexanes, 5/95, v/v). ¹H NMR (CDCl₃) δ 8.40 (bs, 1H, NH), 8.27 (d, J=2.21Hz, 1H, H_Aryl), 7.32 (d, J=8.51Hz, 1H, H_Aryl), 7.05 (dd, J=8.52Hz, J=2.25Hz, 1H, H_Aryl), 2.50 (s, 3H, CH₃); ¹³C NMR (CDCl₃) δ 154.78 (d, JCF=37.82Hz, CO), 139.95 (Ar-N), 132.44 (Ar-S), 130.73 (Ar), 128.84 (Ar-Cl), 126.64 (Ar), 108.01 (NCN), 87.07 (C(CH₃)₃), 27.82 (C(CH₃)₃), 15.85 (S-Me).
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129.32 (Ar), 124.42 (Ar), 119.83 (Ar-Cl), 118.75 (Ar), 115.62 (d, J CF=288.66Hz, CF3), 15.66 (CH3); 19F NMR (CDCl3) δ -75.85 (CF3). MS m/z 269.9 (M+ + H).

2.5.1.49.3.- (2-chloro-5-(methylthio)phenyl)-1-((3-(fluoromethoxy)phenyl)-1-methylguanidine (37).

3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyphenyl)-1-methylguanidine (14) (100 mg, 0.31 mmol) and sodium iodide (47 mg, 0.31 mmol) were added to a stirred suspension of sodium hydride (26 mg, 0.65 mmol) in dimethoxyethane (3 mL) at 0 °C. After 30 minutes bromofluoromethane 2M in DMF (155 µL, 0.31 mmol) was added. After 23 h stirring at room temperature the reaction mixture was poured into water (20 mL) and extracted with dichloromethane (3 x 10 mL). The combined organic fraction was washed with water (10 mL), brine (10 mL), dried with anhydrous MgSO4, filtrated and evaporated to dryness under vacuo. The crude oil was purified over silica with ethyl acetate/petroleum ether (60-80) (67/33, v/v), the title compound as a colorless oil (75 mg, 0.21 mmol, 68%). Rf 0.40 (ethyl acetate/petroleum ether (60-80), 67/33, v/v). 1H NMR (CDCl3) δ 7.38-7.24 (m, 2H, H Aryl), 7.09-7.05 (m, 2H, H Aryl), 6.98-6.95 (m, 1H, H Aryl), 6.90 (d, J=2.29Hz, 1H, H Aryl), 6.80 (dd, J=8.35, J=2.33Hz, 1H, H Aryl), 5.71 (d, J HF=54.32Hz, 2H, FCH2), 3.89 (bs, 2H, NH), 3.40 (bs, 3H, NCH3), 2.44 (s, 3H, SCH3); 13C NMR (CDCl3) δ 157.60 (d, J=2.87Hz, Ar-O), 150.76 (Ar-NCH3), 147.42 (NCN), 146.00 (Ar-NH), 137.81 (Ar-S), 130.72 (Ar-S), 130.14 (Ar), 124.44 (Ar-Cl), 122.50 (Ar), 121.79 (Ar), 121.42 (Ar), 115.37 (d, J CF=1.42Hz, Ar), 114.44 (d, J CF=1.38Hz, Ar), 100.51 (d, J CF=219.67Hz, FCH2), 38.86 (NCH3), 16.04 (SCH3); 19F NMR (CDCl3) δ 149.14 (t, J HF=54.31Hz, 1F, FCH2). The free base was converted into its fumaric acid salt (15 mg, 0.03 mmol, 13%). 1H NMR (DMSO-d6) δ 7.38-7.07 (m, 2H, H Aryl), 7.07-7.05 (m, 2H, H Aryl), 6.93-6.89 (m, 1H, H Aryl), 6.85-6.78 (m, 2H, H Aryl), 6.59 (s, 1.7H, fumaric acid), 5.85 (d, FCH2, J HF=54.31Hz, 2H), 5.74 (bs, 2H, NH), 3.28 (s, 3H, NCH3), 2.43 (s, 3H, SCH3). HRMS calcd for C16H17ClF4FN3OS (M+ + H) 354.0838, found 354.0823. HPLC system A: purity 95.8% tR = 4.22 min.

2.5.1.50.3.- (2-chloro-5-(methylthio)phenyl)-1-((3-(2-fluoroethoxy)phenyl)-1-methylguanidine (38).

The reaction of 3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyphenyl)-1-methylguanidine (14) (322 mg, 1.00 mmol) with 1-fluoro-2-bromoethane (0.25 mL, 3.01 mmol) according to procedure D yielded, after purification over silica with dichloromethane/methanol (95/5, v/v), the title compound as a yellow oil (226 mg, 0.61 mmol, 13%). Rf 0.31 (dichloromethane/methanol, 95/5, v/v). 1H NMR (CDCl3) δ 7.35-7.25 (m, 2H, H Aryl), 7.07-7.05 (m, 2H, H Aryl), 6.93-6.89 (m, 1H, H Aryl), 6.85-6.78 (m, 2H, H Aryl), 6.59 (s, 1.7H, fumaric acid), 5.85 (d, FCH2, J HF=54.31Hz, 2H), 5.74 (bs, 2H, NH), 3.28 (s, 3H, NCH3), 2.43 (s, 3H, SCH3). HRMS calcd for C16H17ClF4FN3OS (M+ + H) 354.0838, found 354.0823. HPLC system A: purity 95.8% tR = 4.22 min.
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159.46 (Ar-O), 151.13 (Ar-NCH3), 146.96 (CN), 145.65 (Ar-NH), 137.82 (Ar-S), 130.56 (Ar), 130.11 (Ar), 124.65 (Ar-Cl), 122.70 (Ar), 121.53 (Ar), 119.69 (Ar), 113.54 (Ar), 112.86 (Ar), 81.87 (d, JCF=171.05 Hz, FCH2CH2O), 77.36 (d, JCF=20.43 Hz, FCH2CH2O), 38.93 (NCH3), 16.01 (SCH3); 19F NMR (CDCl3) δ 16.39 (tt, JHF=47.27 Hz, JHF=27.88 Hz, 1F, FCH2). The free base was converted into its fumaric acid salt (168 mg, 0.37 mmol, 85%). 1H NMR (DMSO-d6) δ 7.32-7.26 (m, 2H, H Aryl), 6.93-6.79 (m, 5H, H Aryl), 4.66 (dt, JHF=47.06 Hz, J=5.75 Hz, 2H, FCH2CH2O), 4.26 (bs, 2H, NH), 4.11 (t, JHF=6.10 Hz, 2H, FCH2CH2O), 3.29 (s, 3H, NCH3), 2.43 (s, 3H, SCH3), 2.10 (dq, JHF=25.80 Hz, J=6.11 Hz, 2H, FCH2CH2O). HRMS calcd for C18H21ClFN3OS (M+ + H) 382.1151, found 382.1134. HPLC system A: purity 99.9%, tR = 4.63 min.

2.5.1.51.3-(2-chloro-5-(methylthio)phenyl)-1-(3-(3-fluoropropoxy)phenyl)-1-methylguanidine (39).

The reaction of 3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyp henyl)-1-methylguanidin (14) (323 mg, 1.04 mmol) with 1-fluoro-3-bromopropane (0.1 mL, 1.09 mmol) according to procedure D yielded, after purification over silica with dichloromethane/methanol (95/5, v/v), the title compound as a brown oil (278 mg, 0.73 mmol, 73%). Rf 0.22 (dichloromethane/methanol, 95/5, v/v). 1H NMR (CDCl3) δ 7.33-7.25 (m, 2H, H Aryl), 6.93-6.79 (m, 5H, H Aryl), 4.66 (dt, JHF=47.06 Hz, J=5.75 Hz, 2H, FCH2CH2O), 4.26 (bs, 2H, NH), 4.11 (t, JHF=6.10 Hz, 2H, FCH2CH2O), 3.43 (s, 3H, NCH3), 2.46 (s, 3H, SCH3), 2.19 (dq, JHF=26.07 Hz, J=5.91 Hz, 2H, FCH2CH2O), 3.29 (s, 3H, NCH3), 2.10 (dq, JHF=25.80 Hz, J=6.11 Hz, 2H, FCH2CH2O). HRMS calcd for C18H21ClFN3OS (M+ + H) 382.1151, found 382.1134. HPLC system A: purity 99.9%, tR = 4.63 min.

2.5.1.52.3-(2-chloro-5-(methylthio)phenyl)-1-(3-(difluoromethoxy)phenyl)-1-methylguanidine ([11C]15).

[11C]CO2 was trapped into a solution of LiAlH4 in THF (0.1 M, 0.1 mL) at room temperature by a helium flow of 10 mL·min⁻¹. The solution was heated to 130 °C and the helium flow was increased to 100 mL·min⁻¹ to evaporate the THF. After 3 min the
helium flow was adjusted to 10 mL·min\(^{-1}\), HI (55% solution, 0.2 mL) was added and \([^{11}\text{C}]\text{CH}_3\text{I}\) was transferred into the reaction vial containing precursor 24 (0.5 mg, 1.40 \(\mu\text{mol}\)), NaOH (5M, 5 \(\mu\text{L}\)) and dimethylformamide (300 \(\mu\text{L}\)). After complete distillation of \([^{11}\text{C}]\text{CH}_3\text{I}\) the reaction mixture was heated at 80 °C for 3 min. The reaction mixture was quenched with 10 mM ammoniumhydrogencarbonate (pH=7.4, 300 \(\mu\text{L}\)) solution before loading it onto preparative HPLC (System C). The product eluted at 6 min, ([\(^{11}\text{C}]26, 21 \text{min}\) was collected and diluted with water (50 mL). The solution was concentrated on a tC18plus Seppak, rinsed with water (20 mL), subsequently eluted with ethanol (96%, 1.5 mL) and diluted with a solution of 7.11 mM NaH\(_2\)PO\(_4\) in 0.9% NaCl (w/v in water), pH 5.2 (13.5 mL) to give a final solution of 9.6% ethanol in phosphate buffer containing \([^{11}\text{C}]\text{15}\). The specific activity was calculated against a calibration curve of 15 using HPLC system B.

2.5.1.53. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(trifluoromethoxy)phenyl)-1-\([^{11}\text{C}]\text{methylguanidine (}[^{11}\text{C}]16\). See method for \([^{11}\text{C}]\text{15}\), using \(25\) as precursor. HPLC retention time of 5 minutes for \([^{11}\text{C}]\text{16}\) and 12 min for \([^{11}\text{C}]\text{27}\).

2.5.1.54. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-\([^{18}\text{F}]\text{fluoromethoxy)phenyl)-1-\text{methylguanidine (}[^{18}\text{F}]37\). After irradiation, \([^{18}\text{F}]\text{fluoride was trapped on a PS-HCO}_3^-\) column. It was eluted from the anion exchange column into a screw cap reaction vessel with 1 mL of acetonitrile/water (9/1, v/v) containing 13 mg (34.5 \(\mu\text{mol}\)) of Kryptofix 2.2.2 and 2 mg (14.5 \(\mu\text{mol}\)) of potassium carbonate. The solution was evaporated to dryness under a helium flow (50 mL·min\(^{-1}\)) and reduced pressure at 90 °C. Acetonitrile (0.5 mL) was added and evaporated again. After cooling to room temperature, dibromomethane/acetonitrile (0.5 mL, 1:1, v/v) was added and heated to 100 °C for 5 minutes. The reaction vessel was cooled to 30 °C and a helium flow (50 mL·min\(^{-1}\)) was used to distil the volatile \([^{18}\text{F}]\text{fluoromethylbromide through a series of 4 coupled Silica Plus Seppaks for purification, followed by a preheated silvertriflate column at 200 °C to the second reaction vessel. The second reaction vessel was precharged with 60% sodium hydride in mineral oil (1.0 mg, 25 \(\mu\text{mol}\)) and precursor (14, 0.5 mg, 1.6 \(\mu\text{mol}\)) dissolved in dry DMF (250 \(\mu\text{L}\)). The solution was heated for 15 minutes at 100 °C before quenching the reaction with 25 mM ammonium dihydrogen phosphate (0.6 mL, pH 2.5) solution. The reaction mixture was purified by semi-preparative HPLC (System E). The product eluted at 26 min, was collected and diluted with water (50 mL). The solution was concentrated on a tC18plus Seppak, rinsed with water (20 mL), subsequently eluted with ethanol (96%, 1.5 mL) and diluted with a solution of 7.11 mM NaH\(_2\)PO\(_4\) in 0.9% NaCl (w/v in water), pH 5.2 (13.5 mL) to give a final solution of
9.6% ethanol in phosphate buffer containing $[^{18}\text{F}]$37. The specific activity was calculated against a calibration curve of 37 using HPLC system D.

2.6. Pharmacology

2.6.1. Animals

For in vivo studies, B6C3F1/J mice were obtained from the Jackson Laboratory (USA) and were housed in groups of four to six per cage until treatment. Animals were kept at a constant temperature of 21 °C and under a 12-h light/dark cycle, in which lights were switched on at 8:00 a.m. Animals had unrestricted access to food (Teklad Global 16% Protein Rodent Diet, Harlan, Madison, WI, USA) and water. All animal experiments were performed in compliance with Dutch laws on animal experimentation and after approval by the local animal ethics committee, study number DEC_PET12-01.

2.6.2. Membrane preparation

Male Wistar rats (150-200 g) were killed by decapitation. The forebrains were rapidly removed and homogenized using a DUALL tissue homogenizer (10 strokes, 2,000 rpm), in a 7-fold excess (v/w) of ice-cold 0.25 M sucrose. The nuclei and cell debris were removed by centrifugation (10 min x 400 x g) in a Sorvall RC-6 refrigerated centrifuge (rotor SA600). The supernatant was decanted and the resulting pellet was rehomogenized in 5 vol 0.25 M sucrose and recentrifuged. The combined supernatants were diluted in Tris-acetate buffer (50 mM, pH 7.4) to a final dilution of 40 v/w, and centrifuged for 30 min x 30,000 x g, in order to obtain membranes from the cell surface, mitochondrial, and microsomal fractions. The pellet was resuspended in 20 vol of 50 mM Tris buffer containing 0.04 % Triton X-100 (pH 7.4), and was kept at 25 °C for 2 hr before recentrifugation. The resulting pellet was suspended in Tris-HCl buffer (dilution 4, pH 7.4) and stored at -80 °C in 5 ml aliquots. On the day of each experiment, membranes were thawed to room temperature and washed twice with Tris buffer by centrifugation (30 min x 48,000 x g). After the final centrifugation step, pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) and further diluted to 40 vol of buffer per g original weight wet tissue for competition binding experiments. Protein concentration was determined using the BCA protein kit (Sigma-Aldrich, The Netherlands).
2.6.3. Competition binding assays

*In vitro* competition binding experiments were performed using 5 nM \(^{3}\text{H}\)MK-801 (specific activity 22.5 Ci/mmol; PerkinElmer, USA). All compounds were dissolved as 10 mM stock solutions in DMSO, and used in a concentration range from \(10^{-4}\) to \(10^{-12}\) M, with a DMSO concentration of maximal 1%. Competition binding experiments were conducted at room temperature, in a final volume of 500 µL assay buffer (50 mM Tris-HCl, pH 7.4), containing 1 µM L-glutamate and glycine. The incubation mixture was composed of 400 µL membrane suspension (protein concentration 1 µg·µL\(^{-1}\)), 50 µL \(^{3}\text{H}\)MK-801, 45 µL assay buffer and 5 µL unlabeled drug solution. Nonspecific binding was determined in the presence of 30 µM GMOM (5). Incubations were terminated after 17 hr by filtration, using a 48-well Brandel harvester and Whatman GF/B filters, presoaked in 0.3% polyethyleneimine. The filters were washed three times with 3 mL of ice-cold Tris-HCl buffer (pH 7.4), and subsequently radioactivity was determined by liquid scintillation spectrometry in 5 mL of Optiphase-HiSafe 3, at an efficiency of 40%.

2.6.4. Data analysis

\(K_i\) values were determined by nonlinear regression analysis using the equation:

\[
\log EC_{50} = \log(10^{\log K_i \times (1 + \text{Radioligand}_{\text{NM}}/\text{Hot}_{\text{Kd}_{\text{NM}}})})
\]

(GraphPad Software Inc., San Diego, CA).

2.6.5. Determination of LogDoct,7.4

The distribution of the radiolabeled compounds between 1-octanol and 0.2M phosphate buffer (pH = 7.4) was measured in triplicate at room temperature. Briefly, 1 mL of a 20 MBq·mL\(^{-1}\) solution of the radiolabeled compound in 0.2M phosphate buffer (pH 7.4) was vigorously mixed with 1 mL of 1-octanol for 1 min at room temperature using a vortex. After a settling period of 30 min, five samples of 100 µL were taken from both layers. For determining recovery, 5 samples of 100 µL were taken from the 20 MBq·mL\(^{-1}\) solution. All samples were counted for radioactivity. The LogDoct,7.4 value was calculated according to LogDoct,7.4 = 10Log(A\(_{\text{oct}}\) / A\(_{\text{buffer}}\)), where A\(_{\text{oct}}\) and A\(_{\text{buffer}}\) represent average radioactivity of 5 1-octanol and 5 buffer samples, respectively.
2.6.6. Metabolite analysis

The metabolic profile of the labeled compounds was studied in male, 7-9 weeks old, B6C3 mice. Animals were anaesthetized with an i.p. injection of Hypnorm/dormicum (12 mL·kg⁻¹), after followed by injecting 80-125 MBq of labeled compound in the tail vein. Mice were sacrificed at 5, 15, 30 and 60 minutes. Immediately, 0.5 mL of blood was collected via a heart puncture, and the brain was removed. Blood was collected in a heparin tube and centrifuged for 5 min at 4,000 rpm (Hettich universal 16, Depex B.V., the Netherlands). Plasma was separated from blood cells and loaded onto a tC2 Sep-Pak (Waters, the Netherlands) and the Sep-Pak was washed with 3 mL of water. This eluate was defined as the polar radiolabeled metabolite fraction. Subsequently, the tC2 Sep-Pak was eluted with 2 mL of methanol and 1 mL of water. This eluate was defined as the nonpolar fraction and was analyzed using HPLC (system F). Brain tissue was homogenized with an ultrasonic homogenizer (Braunsonic 1510, Germany) in 10 mL of cold water, under ice cooling, and subsequently centrifuged at 4,000 rpm for 5 min. Separated supernatants were loaded onto a tC2 Sep-Pak and first washed with 10 mL of water to obtain the polar fraction and then washed with 2 mL of methanol and 1 mL of water to obtain the nonpolar fraction which was analyzed by HPLC (system F).

2.6.7. Biodistribution studies

Male, 7-9 weeks old, B6C3 mice were used. Animals were anaesthetized with an i.p. injection of Hypnorm/dormicum (12 mL·kg⁻¹), after which [¹¹C]¹⁵ (24.4 ± 3.5 MBq at t₀) or [¹⁸F]³⁷ (19.0 ± 1.1 MBq at t₀) was administered via the tail vein, in a solution of 7.11 mM NaH₂PO₄ in 0.9% NaCl (w/v in water) containing 9.6% ethanol (5 mL·kg⁻¹). Following these injections, mice were killed by cervical dislocation at 5, 10, 30, or 60 min (n=4-6). At each time point, blood was obtained by heart punctures and selected organs, including heart, liver, kidneys, lungs and brain were removed. The brain was further dissected into prefrontal cortex, striatum, cerebral cortex, hippocampus and cerebellum. All organs and brain areas were weighed, and recovered radioactivity was determined using a Compugamma (LKB Wallac), with 5 x 10 μL aliquots of the injected formulation as standard. Results are expressed as the standardized uptake value (SUV): (cpm recovered/g tissue)/(cpm injected/g body weight). Two-way repeated measures ANOVA, followed by LSD post-hoc analysis was used for between-region (or organ) comparisons of radiotracer uptake at different time points.
2.6.8. Ex vivo autoradiography & blocking study

To visualize the distribution and specificity of \[^{11}C\]15 or \[^{18}F\]37 for NMDAr, anaesthetized mice were injected with either saline or MK-801 (0.6 mg·kg\(^{-1}\), intraperitoneal injection), 10 min before a tail-vein injection of each of the radiolabeled compounds (n=2). 5 min following injection of \[^{11}C\]15 or 15 min after \[^{18}F\]37 injection, mice were killed by cervical dislocation, and their brains were removed, frozen in liquid nitrogen and processed for quantitative autoradiography. All autoradiographic procedures, including animal dosing, tissue processing and X-ray film handling, were conducted in parallel for control and MK-801 treated mice. Briefly, 20 μm coronal brain sections were cut at 300 μm intervals, from rostral to caudal areas. Sections from each mouse were exposed to Kodak Biomax MR-1 film. Films were developed after 24 hr, and relevant optical density (ROD) values were obtained using MCID software. Sections from control and MK-801 treated mice were processed in parallel. All brain regions were identified by reference to the mouse atlas of Franklin and Paxinos (2001).

2.7. Acknowledgements

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project LeARN (grant 02N-101). We would like to acknowledge Ester Kooijman, Emily Nash, Henri Greuter, Kevin Takkenkamp and Marissa Rongen for their assistance during the experiments.
2.8. Supplementary data

Figure S1. HPLC chromatograms of $[^{11}C]15$. The left hand side shows the semi-preparative purification (System C), the right hand side shows the quality control (System B). Both upper panels represent the UV-channel and the lower panels the radioactivity channel.

Figure S2. HPLC chromatograms of $[^{18}F]37$. The left hand side shows the semi-preparative purification (System E), the right hand side shows the quality control (System D). Both upper panels represent the UV-channel and the lower panels the radioactivity channel.
Figure S3. Differences in binding of $[^{11}\text{C}]15$ in 5 brain regions, with on the left side normal binding and on the right after pre-treatment with MK-801 (0.6 mg·kg$^{-1}$).
Table S1. Tested conditions for preparing $N,N'$-protected-$N'$ precursors 40 and 41.

| Entry | Cmp. | R<sub>1</sub> | R<sub>2</sub> | Cmp. | Solvent<sup>b</sup> | Temp.<sup>c</sup> | Time<sup>d</sup> | Product | Yield<sup>e</sup> |
|-------|------|-------------|-------------|------|---------------------|-----------------|----------------|---------|----------------|------------------|
| 1     | 35   | Boc         | CN          | 29h  | H                   | CB              | 165            | 6       | 40             | 0                |
| 2     | 35   | Boc         | CN          | 29h  | H                   | CB              | 165            | 6       | 40             | 0                |
| 3     | 35   | Boc         | CN          | 29h  | H                   | HFIP            | 70             | 16      | 40             | 1                |
| 4     | 32   | Boc         | H           | 30h  | CN                  | HFIP            | 70             | 6       | 40             | 0                |
| 5     | 36   | Tfa         | H           | 30h  | CN                  | HFIP            | 120            | 8       | 41             | 0                |
| 6     | 36   | Tfa         | H           | 30h  | CN                  | CB              | 165            | 6       | 41             | 0                |

<sup>a</sup> Compound, <sup>b</sup> CB: chlorobenzene, HFIP: hexa-fluoro isopropanol, <sup>c</sup> Temperature (°C), <sup>d</sup> hours, <sup>e</sup> (%).

Figure S4. Differences in binding of $[^{18}F]$37 in 5 brain regions, with on the left side normal binding and on the right after pre-treatment with MK-801 (0.6 mg·kg⁻¹).
2.9. References


Synthesis, structure activity relationship, radiolabeling and preclinical evaluation of high affinity ligands for the ion channel of the N-methyl-D-aspartate receptor as potential imaging probes for positron emission tomography


Chapter 3.

Synthesis, radiolabeling and evaluation of novel amine guanidine derivatives as potential positron emission tomography tracers for the ion channel of the N-methyl-D-aspartate receptor

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Abstract

The N-Methyl-D-Aspartate receptor (NMDAr) is involved in many neurological and psychiatric disorders including Alzheimer’s disease and schizophrenia. The aim of this study was to develop a positron emission tomography (PET) ligand to assess the bioavailability of the NMDAr ion channel in vivo. A series of tri-N-substituted diarylguanidines was synthesized and their in vitro binding affinities for the NMDAr ion channel assessed in rat forebrain membrane fractions. Compounds 59, 61 and 64 were radiolabeled with either carbon-11 or fluorine-18 and ex vivo biodistribution and metabolite studies were performed in Wistar rats. Biodistribution studies showed high uptake especially in prefrontal cortex and lowest uptake in cerebellum. Pretreatment with MK-801, however, did not decrease uptake of the radiolabeled ligands. In addition, all three ligands showed fast metabolism.
3.1. Introduction

Excitatory neurotransmission in the mammalian central nervous system (CNS) is primarily accountable to the glutamate receptor system. Glutamate receptors are divided into ionotropic and metabotropic receptors. Ionotropic receptors are subdivided into three groups of receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate receptor (NMDAr) [1]. NMDArs are necessary for long term potentiation, and are thought to play an important role in learning and memory [2]. It is hypothesized that hypoactivity of the NMDAr, associated with advancing age, may have a role in developing Alzheimer’s disease and other neurodegenerative diseases [3,4]. Imaging of NMDArs using positron emission tomography (PET) could make it possible to assess the NMDAr status in vivo. To date, only $[^{18}\text{F}]$-GE-179 has been used successfully in human applications [5].

The NMDAr is a heteromultimeric assembly of four subunits. To date, three types of subunits are known: the NR1 subunit with eight splice variants (NR1a-h) [6], the NR2 subunit encoded by four distinct genes (NR2A-D) [7], and the NR3A and NR3B subunits (2 genes) [8]. This assembly together forms the ion channel of the NMDAr.

Recently, several structural classes with NMDAr affinity were reported. Propargylamine and acetylene conjugated polycyclic cage derivatives, β- and γ-carboline derivatives, pentamidine analogs of MK-801 and ifenprodil and (3-hydroxy-pyrazolin-5-yl)glycine based ligands. Unfortunately, all exhibit binding affinities in the micro-molar range [9-12].

Non-competitive ion channel NMDAr antagonists, such as [5R,10S]-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), N-(1-[thienyl]cyclohexyl)piperidine (TCP) and ketamine are termed “use-dependent” ligands. These non-competitive antagonists gain access to the ion channel site primarily when the NMDAr is in an open state [13,14], i.e. they can bind within the channel when the receptor is activated by the agonist glutamate and the co-agonist glycine. Therefore, binding of non-competitive antagonists is proportional to the amount of activated, open receptor channels. Hence, by using non-competitive radiolabeled NMDAr antagonists and PET, the status of regional activation of NMDArs can be assessed in vivo.

The class of guanidine compounds with affinity for the NMDAr ion channel is known since 1989 [15–23]. Structure activity relationships studies showed that 5 (GMOM, $K_i$: 5.2 – 21.7 nM against $[^{3}\text{H}]$MK801) [22,24], 6 (CNS 5161, $K_i$: 1.87 nM against $[^{3}\text{H}]$MK801) [20] and 7 (GE-179, $K_i$: 2.4 nM against $[^{3}\text{H}]$TCP) [5] have superior $K_i$ values towards the ion channel of the NMDAr (Figure 1). Apart from its $K_i$ value,
ligand 5 showed promising characteristics for use as an NMDA PET tracer, although human applications have not been reported yet. $[^{11}\text{C}]6$ has been used in patients with Parkinson’s disease, but no significant increase in uptake of $[^{11}\text{C}]6$ was seen in patients as compared with control subjects [25]. Recently, in an initial evaluation of $[^{18}\text{F}]7$ in healthy volunteers high and rapid brain extraction was observed, with a relatively homogeneous distribution in grey matter and rapid peripheral metabolism. Nevertheless, quantification of $[^{18}\text{F}]7$ appeared to be feasible [5].

Figure 1. Structures of radiolabeled N,N’-diaryl-N-methylguanidines.

So far, there have been no reports on meta substituted alkylamine analogues of 5-7. Therefore, the aim of this study was to synthesize and evaluate amine and pyridine analogs of 5 and 6 as potential PET ligands for the NMDAr ion channel. Synthesis, binding affinity to the ion channel of the NMDAr, radiolabeling of higher affinity compounds, the LogD$_{7.4}$ value, metabolism and biodistribution in rats were investigated.

3.2. Results and discussion

3.2.1. Chemistry

The synthesis of the pyridine substituted guanidines (45a, c) is shown in Scheme 1. The N-methyl-N-(pyridinyl)cyanamides 44a-c were synthesized by a three step procedure adapted from Stanovnik et al [26] and Huntsman et al [27]. First, the formamide oximes (43a-c) were obtained by a two step reaction from the aminopyridines 42a-c with N,N-dimethylformamide dimethyl acetal (DMF-DMA) in 2-propanol, followed by treatment with hydroxylamine hydrochloride (NH$_2$OH·HCl) in 52-71% yield. Secondly, a reaction with DMF-DMA in toluene yielded the N-methylcyanamides 44a-c in 30-61% yield. The pyridine guanidines 45a, c were synthesized by condensation of 2-chloro-5-(methylthio)aniline hydrochloride in chlorobenzene with 44a-c in 34-39% yield, but 45b could not be obtained using this method. The reason for this is unclear, presumably the meta configuration of the pyridine ring deactivates the cyanic moiety too much for condensation. As compound
45a and 45c showed affinities less than 10 µM towards the NMDA receptor, no further research was performed to obtain compound 45b.

Scheme 1. Synthesis of pyridine substituted guanidines 45a, c.

Unsubstituted aminophenyl guanidine 52 was synthesized according to Scheme 2 via condensation of 50 with 2-chloro-5-(methylthio)aniline hydrochloride in 46% yield and subsequent quantitative deprotection of the 2,5-dimethylpyrrole group using NH₂OH·HCl [28]. Compound 50 was synthesized in 4 steps from 3-nitro-aniline (46) in 64% yield. First, 46 was protected with a 2,5-dimethylpyrrole group [29] followed by a reduction of the nitro moiety [30]. Secondly, amine 48 was reacted with cyanogen bromide [31] and then methylated to obtain 50.

Scheme 2. Synthesis of amino guanidine 52.

The alkyl substituted amine guanidines 59, 61, 64 and 67 were prepared using 55-57 as key intermediates. Amine 55 was mono protected to prevent double alkylation in the synthesis of 57 and 65, and to preclude formation of side products during the
guanidine formation of 61 and 67. Scheme 3 shows the synthesis of mono- and dimethylaminophenyl guanidines 59 and 61. The N-(3-aminophenyl)-N-methylcyanamide intermediates 55-57 were prepared from 3-nitro-aniline 46. Cyanamide 53 was obtained in 93% yield [32]. After methylation of the cyanamide moiety and reduction of the nitro moiety, 55 was obtained in 72% overall yield. Subsequent monoprotection of the amine moiety with a trifluoroacetic acid group [33] gave 56 in 86% yield and the subsequent methylation gave 57 in quantitative yield. Compound 55 was dimethylated to obtain 58 in 51% yield. The condensation of 58 with 2-chloro-5-(methylthio)aniline hydrochloride in chlorobenzene gave dimethylaminophenyl guanidine 59 in 59% yield. Synthesis of N-trifluoroacetic-N-methylaminophenyl guanidine 60 was carried out using the trifluoroacetyl protected cyanamide 57 in 41% yield. Deprotection of 60 under basic aqueous conditions yielded 61 quantitatively.

Scheme 3a. Synthesis of mono- and dimethyl amino guanidine 59 and 61.

Reagents and conditions: (a) CNBr, Acetic acid/H2O, 1M NaOH, 0 °C – RT, 20h; (b) K2CO3, CH3I, DMF, 45 °C, 45 min; (c) acetic acid, Fe, EtOH/H2O, reflux, 10 min; (d) TFAA, DCM, 0 °C, 1h; (e) K2CO3, CH3I, DMF, 45 °C, 2.5h; (f) K2CO3, CH3I, DMF, 45 °C, 1 h; (g) 2-chloro-5-(methylthio)aniline hydrochloride, chlorobenzene, 165 °C, 7 h; (h) K2CO3, MeOH/H2O, RT, 1 h.
Intermediate 57 was deprotected and alkylated with fluoroethylbromide to obtain 63 in 30% overall yield (Scheme 4). N-methyl-N-fluoroethyl guanidine was synthesized in 53% yield. However, by using standard conditions to obtain guanidines 66 and 64, namely 165 °C in chlorobenzene, an inseparable side product was formed. High resolution mass spectrometry and 13C-NMR measurements revealed an impurity containing a chloroethyl moiety besides the fluoroethyl product. Formation of this chlorinated side product was circumvented by lowering the temperature to 140 °C and using toluene instead of chlorobenzene as solvent.

Scheme 4a. Synthesis of fluoroethylmethyl amino guanidine 64.

![Scheme 4a](image)

a Reagents and conditions: (a) K₂CO₃, MeOH/H₂O, RT, 1 h; (b) K₂CO₃, KI, Br(CH₂)₂F, DMF/THF, 80 °C, 24 h; (c) 2-chloro-5-(methylthio)aniline hydrochloride, toluene, 140 °C, 8 h.

The fluoroethylamine analogue 67 was synthesized via a similar approach, starting from 56 (Scheme 5). Alkylation with fluoroethylbromide provided 65 in 70% yield. Subsequent condensation with 2-chloro-5-(methylthio)aniline hydrochloride in toluene (66, 66%) and quantitative deprotection led to fluoroethylamine guanidine 67.


![Scheme 5a](image)

a Reagents and conditions: (a) K₂CO₃, KI, Br(CH₂)₂F, DMF/THF, 80 °C, 26 h; (b) 2-chloro-5-(methylthio)aniline hydrochloride, toluene, 140 °C, 6.5 h; (c) K₂CO₃, MeOH/H₂O, RT, 1 h.

3.2.2. Structure activity relationship study

Table 1 summarizes binding affinities of the synthesized guanidines for the ion channel of the NMDAr. The binding affinity was determined by measuring the ability
of various concentrations of unlabeled ligand to inhibit specific binding of [\( ^3 \text{H} \)]MK-801 to rat forebrain plasma membranes. Substitution of a phenyl group (9) by a pyridyl group (45a, c) was not allowed as the affinity of the guanidines 45a and c dropped to values above 10 \( \mu \text{M} \).

The introduction of an amine rather than hydroxyl moiety, however, was allowed in view of binding affinity towards the ion channel of the NMDAr. Analogous to the improvement in affinity of 5 by replacing the hydroxyl moiety with methoxy 14, a similar phenomenon was observed after monomethylation of 52. The binding affinity of 52 increased 23 fold to 19.1 nM for 61. Double methylation increased the affinity even further to 1.35 nM for compound 59. Introduction of a fluoroethyl moiety in 67 increased the affinity, compared with 52, 9 fold. Another 10 fold increase to 4.81 nM for 64 was reached when a methyl group was introduced. These results suggest that a tertiary amine, substituted with short alkyl groups, is favored over a secondary amine with respect to binding affinity for the ion channel of the NMDAr. From the short alkyl groups, a methyl substitution (59, 61) is preferred over a fluoroethyl substitution (64, 67) as the corresponding binding affinity is about 3 fold higher.

### Table 1. Affinity (nM) to the ion channel of the NMDAr.

<table>
<thead>
<tr>
<th>Compound&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Y</th>
<th>Z</th>
<th>R</th>
<th>( K_i ) (nM)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C</td>
<td>C</td>
<td>H</td>
<td>307 ± 130</td>
</tr>
<tr>
<td>45a</td>
<td>N</td>
<td>C</td>
<td>H</td>
<td>&gt; 10 ( \mu \text{M} )</td>
</tr>
<tr>
<td>45c</td>
<td>C</td>
<td>N</td>
<td>H</td>
<td>&gt; 10 ( \mu \text{M} )</td>
</tr>
<tr>
<td>14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C</td>
<td>C</td>
<td>OH</td>
<td>551 ± 100</td>
</tr>
<tr>
<td>5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C</td>
<td>C</td>
<td>OCH(_3)</td>
<td>21.7 ± 7.52</td>
</tr>
<tr>
<td>52</td>
<td>C</td>
<td>C</td>
<td>NH(_2)</td>
<td>439 ± 70.6</td>
</tr>
<tr>
<td>61</td>
<td>C</td>
<td>C</td>
<td>NH(_2)CH(_3)</td>
<td>19.1 ± 7.76</td>
</tr>
<tr>
<td>59</td>
<td>C</td>
<td>C</td>
<td>N(CH(_3))(_2)</td>
<td>1.35 ± 0.32</td>
</tr>
<tr>
<td>67</td>
<td>C</td>
<td>C</td>
<td>NH(CH(_2))(_2)F</td>
<td>48.3 ± 22.6</td>
</tr>
<tr>
<td>64</td>
<td>C</td>
<td>C</td>
<td>NCH(_3)(CH(_2))(_2)F</td>
<td>4.81 ± 2.63</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fumaric acid salt, 45c, 14: free base; <sup>b</sup> \( K_i \) measured against 5 nM [\( ^3 \text{H} \)]MK-801 presented as mean ± SD of 3 independent determinations, each conducted in triplicate; <sup>c</sup> \( K_i \) values taken from Klein et al [24].

### 3.2.3. Radiochemistry

Compounds 59, 61 and 64 were radiolabeled with either carbon-11 or fluorine-18.
3.2.3.1. Synthesis of $^{[11]}\text{C}59$

The carbon-11 labeling agent $^{[11]}\text{C}\text{CH}_3\text{I}$ was prepared by reacting cyclotron produced $^{[11]}\text{C}\text{CO}_2$ with lithium aluminium hydride in THF, which subsequently was reacted with hydroiodic acid in radiochemical yields of up to 90% decay corrected (DC) [34,35] Radiolabeling of $^{[11]}\text{C}59$ is depicted in Scheme 6.

Scheme 6. Synthesis of carbon-11 labeled guanidines $^{[11]}\text{C}61$ and $^{[11]}\text{C}59$ using $^{[11]}\text{C}$methyl iodide or $^{[11]}\text{C}$methyl triflate.

Initially, $^{[11]}\text{C}\text{CH}_3\text{I}$ was used as labeling synthon exploring several common base solvent combinations at 80 °C and 3 min reaction time (Table 2, entries 1-6). Unfortunately, yields obtained were below 4% (radiochemical yield, DC). When the more reactive $^{[11]}\text{C}\text{CH}_3\text{OTf}$ labeling synthon was used, radiochemical yields increased.

Table 2. Radiolabeling optimization of $^{[11]}\text{C}59$.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Labeling agent</th>
<th>Solvent</th>
<th>Base</th>
<th>Amount of base</th>
<th>Time</th>
<th>Temp</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH3I</td>
<td>DMSO</td>
<td>5M NaOH</td>
<td>25</td>
<td>3</td>
<td>80</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>CH3I</td>
<td>DMSO</td>
<td>K2CO3</td>
<td>72</td>
<td>3</td>
<td>80</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>CH3I</td>
<td>DMSO</td>
<td>40% TBAOH</td>
<td>7.5</td>
<td>3</td>
<td>80</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>CH3I</td>
<td>DMF</td>
<td>5M NaOH</td>
<td>25</td>
<td>3</td>
<td>80</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>CH3I</td>
<td>DMF</td>
<td>K2CO3</td>
<td>72</td>
<td>3</td>
<td>80</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>CH3I</td>
<td>MeCN</td>
<td>K2CO3</td>
<td>72</td>
<td>3</td>
<td>80</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>7</td>
<td>CH3OTf</td>
<td>DMF</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>80</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>CH3OTf</td>
<td>Acetone</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>80</td>
<td>37.2 ± 10.2</td>
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<tr>
<td>9</td>
<td>CH3OTf</td>
<td>MeCN</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>80</td>
<td>39.5</td>
</tr>
<tr>
<td>10</td>
<td>CH3OTf</td>
<td>MeCN</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>80</td>
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<td>-</td>
<td>-</td>
<td>1</td>
<td>60</td>
<td>46.5</td>
</tr>
<tr>
<td>14</td>
<td>CH3OTf</td>
<td>MeCN</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>40</td>
<td>45.5 ± 6.7</td>
</tr>
<tr>
<td>15</td>
<td>CH3OTf</td>
<td>MeCN</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>30</td>
<td>46.7</td>
</tr>
<tr>
<td>16</td>
<td>CH3OTf</td>
<td>MeCN</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>20</td>
<td>43.2</td>
</tr>
<tr>
<td>17</td>
<td>CH3OTf</td>
<td>Acetone</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>40</td>
<td>31.5</td>
</tr>
</tbody>
</table>

*a used 0.5-0.8 mg (1.6-2.5 µmol) of precursor (61), b 300 µL, c µmol, d minutes, e °C, f Entries 1-8, 14: n=2 presented as mean ± SD, entries 9-13, 15-17: n=1, g Radiochemical yield determined using radio HPLC (Waters µBondapak, C18 10µm 125A, 250 x 4.6mm, 10mM ammonium hydrogen carbonate pH=7.4 / MeCN, 30/70, v/v, 1.5 mL·min⁻¹).
to 47% (Table 2, entries 7-17). $^{[11}\text{C}]\text{CH}_3\text{OTf}$ was prepared by transferring $^{[11}\text{C}]\text{CH}_3\text{I}$ through a column containing silver triflate impregnated Graphpac heated at 200 °C [36]. Further optimization of time, reaction temperature and solvent resulted in optimal reaction conditions of 40 °C and 1 min reaction time in acetonitrile using 0.5-0.8 mg (1.6-2.5 µmol) of precursor 61.

After preparative HPLC purification (Figure S1, Supplementary data), and formulation into a sterile solution, $^{[11}\text{C}]$59 was obtained in 30.7 ± 12.8% (n=14) radiochemical yield, DC, calculated from $^{[11}\text{C}]\text{CH}_3\text{I}$. The radiochemical purity was higher than 96.1% and specific activity was 105 ± 38.8 GBq·µmol⁻¹ at end of synthesis. The total synthesis time of $^{[11}\text{C}]$59 was 22 min.

3.2.3.2. Synthesis of $^{[11}\text{C}]$61

Given the optimal radiolabeling conditions for $^{[11}\text{C}]$59, only time and temperature for the radiosynthesis of $^{[11}\text{C}]$61 were investigated (Scheme 6). Table 3 summarizes radiochemical yields and illustrates that optimal conditions were identical to those used for the synthesis of $^{[11}\text{C}]$59.

Table 3. Radiolabeling optimization of $^{[11}\text{C}]$61.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Labeling agent</th>
<th>Solventb</th>
<th>Timec</th>
<th>Tempd</th>
<th>Yielde,f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH₃OTf</td>
<td>MeCN</td>
<td>1</td>
<td>30</td>
<td>33.1 ± 13.6</td>
</tr>
<tr>
<td>2</td>
<td>CH₃OTf</td>
<td>MeCN</td>
<td>1</td>
<td>40</td>
<td>38.3 ± 9.01</td>
</tr>
<tr>
<td>3</td>
<td>CH₃OTf</td>
<td>MeCN</td>
<td>1</td>
<td>60</td>
<td>34.3 ± 10.6</td>
</tr>
<tr>
<td>4</td>
<td>CH₃OTf</td>
<td>MeCN</td>
<td>3</td>
<td>40</td>
<td>32.1 ± 13.0</td>
</tr>
<tr>
<td>5</td>
<td>CH₃OTf</td>
<td>MeCN</td>
<td>5</td>
<td>40</td>
<td>35.4 ± 5.49</td>
</tr>
</tbody>
</table>

a used 0.5-0.9 mg (1.5-2.7 µmol) of precursor (52), b 300 µL, c minutes, d °C, e Entry 2: n=4, entries 1, 3-5 n=2 presented as mean ± SD, f Radiochemical yield determined using radio HPLC (Alltima C18, 5µm, 250 x 4.6mm, 20mM potassium hydrogen phosphate pH=6.7 / MeOH / MeCN, 20/20/60, v/v/v, 1.0 mL·min⁻¹).

After preparative HPLC purification (Figure S2, Supplementary data), and formulation in a sterile solution, $^{[11}\text{C}]$61 was obtained in 35.6 ± 4.1% (n=6) radiochemical yield, DC, calculated from $^{[11}\text{C}]\text{CH}_3\text{I}$. The radiochemical purity was higher than 95.4% and specific activity was 201 ± 57.0 GBq·µmol⁻¹ at end of synthesis. The total synthesis time of $^{[11}\text{C}]$61 bombardment was 22 min.

3.2.3.3. Synthesis of $^{[18}\text{F}]$64

Initially, compound 64 (Scheme 7) was supposed to be radiolabeled using $^{[18}\text{F}]$fluoroethyltriflate ($^{[18}\text{F}]$71) as labeling synthon in a manner analogous to the synthesis of $^{[11}\text{C}]$59 and $^{[11}\text{C}]$61. First, 2-bromoethyl tosylate 69 was synthesized by
tosylation of 2-bromoethanol in dichloromethane in 31% yield. After nucleophilic substitution with fluorine-18 of 69 in acetonitrile, $[^{18}\text{F}]70$ was distilled from the reaction mixture through a heated silvertriflate/Graphpac column, using a stream of helium. Intermediate $[^{18}\text{F}]70$ was converted online into the more reactive triflate intermediate $[^{18}\text{F}]71$ [37]. Synthon $[^{18}\text{F}]71$ was trapped in the next reaction vessel containing precursor 59 in $33 \pm 13\%$ yield DC. After complete trapping of $[^{18}\text{F}]71$, the coupling reaction was carried out (Scheme 9). For this reaction several reaction conditions were explored. As solvents acetonitrile, DMF or DMSO were used, and as bases potassium carbonate, sodium hydride or tetra-(n-butyl)ammonium hydroxide. The reaction temperature was between 100 and 140 °C, and reaction times between 10 and 20 min. The best conditions, 0.5 mg (1.4 µmol) precursor 59, 300 µL acetonitrile, no base at 100 °C for 10 min, resulted in a radiochemical yield of $7.2 \pm 3.4\%$ yield (N=11, determined on analytical HPLC, Alltima C18, 5 µm, 250 x 4.6 mm, 20 mM potassium hydrogen phosphate pH=6.7 / methanol / acetonitrile, 20/20/60, v/v/v, 1.0 mL·min$^{-1}$). After semi preparative HPLC purification (Reprosphere 100-C18-DE, 5 µm, 10 mM ammonium hydrogen carbonate pH=7.4 / acetonitrile, 30/70, v/v, 4 mL·min$^{-1}$), the overall yield was less than 3% (n=6, DC) with a radiochemical purity of 84-99%.

Scheme 7a. Synthesis of fluorine-18 labeled compound $[^{18}\text{F}]64$.

As the synthesis of $[^{18}\text{F}]64$ did not proceed well using the 2-step alkylation reaction, precursor 74 was synthesized. The tosyl precursor was synthesized in 4 steps starting from intermediate 62 (Scheme 8). First, the hydroxyethyl cyanamide 72 was prepared by alkylation of 62 with bromoethanol in 43% yield, which subsequently was condensed to guanidine 73 in 52% yield. The tosyl leaving group was introduced using standard conditions in 24% yield.
Scheme 8a. Synthesis and fluorination of tosylate precursor 74.

Reagents and conditions: (a) DIPEA, KI, Br(CH2)OH, DMF/THF, 125 °C, 6 h; (b) 2-chloro-5-(methylthio)aniline hydrochloride, toluene, 140 °C, 8 h; (c) Et3N, DMAP, p-toluenesulfonyl chloride, DCM, RT, 21 h; (d) K2CO3, Kryptofix, MeCN, 18F-, 80 °C, 30 min.

Using standard labeling conditions only a limited number of optimizations of the synthesis of [18F]64 was performed (Table 4). The nucleophilic fluorination reaction was carried out using Kryptofix 2.2.2 and potassium carbonate in acetonitrile or DMSO. Variations were made in temperature (80, 100 or 120 °C) and time (10, 20 and 30 minutes). Radiolabeling yield was determined using radio TLC. Labeling yield in DMSO was relatively low, below 10%. Using acetonitrile, however, the yield increased to 43% in 20 minutes at 120 °C. To prevent possible pressure buildup in the reaction vial at higher temperatures, 30 minutes at 80 °C were used.

Table 4. Radiolabeling optimization of [18F]64.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent b</th>
<th>Time c</th>
<th>Yield d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>1</td>
<td>DMSO</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>DMSO</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>DMSO</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>MeCN</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>MeCN</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>MeCN</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>

a 2.5 mg (4.7 µmol) of precursor (74) used, b 500 µL, c minutes, d Radiochemical yield in %, determined using radio TLC (dichloromethane / methanol 95/5, v/v); n=1, e °C.

After preparative HPLC purification (Figure S3, Supplementary data), and formulating into a sterile solution, [18F]64 was obtained in 8.7 ± 1.7% (n=5) radiochemical yield, DC. The radiochemical purity was 96.3 ± 2.3% and specific activity was 78.2 ± 32.0 GBq·µmol⁻¹ at end of synthesis. The total synthesis time of [18F]26 was 90 min.
3.2.4. Determination of lipophilicity expressed as LogD\(_{\text{oct},7.4}\) of 59, 61 and 64

The distribution coefficients of 59, 61 and 64 were measured using their radiolabeled analogues. The lipophilicity was determined using a mixture of phosphate buffer at pH=7.4 and 1-octanol [38]. The LogD\(_{\text{oct},7.4}\) values are shown in Table 5. Although measured LogD\(_{\text{oct},7.4}\) values were not in the optimal range of 2.0 - 3.5 [39], biodistribution studies showed that all ligands crossed the blood brain barrier.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogD(_{\text{oct},7.4})(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^{11}\text{C}]59)</td>
<td>1.62 ± 0.01</td>
</tr>
<tr>
<td>([^{11}\text{C}]61)</td>
<td>1.24 ± 0.00</td>
</tr>
<tr>
<td>([^{18}\text{F}]64)</td>
<td>1.51 ± 0.00</td>
</tr>
</tbody>
</table>

\(^a\)n=3 presented as mean ± SD.

3.2.5. Metabolite analysis

It is important to characterize the metabolic profile of a PET tracer, as radiolabeled metabolites might interfere with quantification of a PET study, especially if those metabolites cross the blood-brain barrier [40].

Metabolites were determined in awake male Wistar rats at 15 and 45 minutes after injection. As potential metabolic pathway de-alkylation of S-methyl, guanidine N-methyl and aniline N-methyl moieties was expected. De-alkylation of the carbon-11 radiolabeled moiety (compound \([^{11}\text{C}]59\) and \([^{11}\text{C}]61\) would lead to radiolabeled polar metabolites, which could interfere with PET quantification, and unlabeled non-polar metabolites, which do not interfere with PET quantification. These radiolabeled polar metabolites most likely represents single carbon molecules such as formaldehyde, formic acid or carbon dioxide, showing no retention on HPLC. In case of \([^{18}\text{F}]64\), the polar metabolite fraction most likely represent the fluoroethyl residue.

Demethylation of the non radiolabeled moieties, however, would lead to radiolabeled metabolites that could interfere with PET imaging, as they may cross the blood-brain barrier and bind to the same or other molecular targets. Non-polar metabolites represent, besides the parent compound, the fractions showing retention on HPLC. Figure 2 shows the metabolic profiles of \([^{11}\text{C}]59\), \([^{11}\text{C}]61\) and \([^{18}\text{F}]64\) as function of time.

45 min after injection of \([^{11}\text{C}]59\), 19% of measured radioactivity in plasma could be attributed to parent compound, and in the brain this was 33%. The decrease in parent fraction in the brain from 62 to 33% between 15 and 45 min indicates fast metabolic degradation. HPLC analysis revealed, next to parent compound, three radiolabeled
non-polar metabolites, most likely structural analogs of \([^{11}\text{C}]59\). Indeed one of these was identified on HPLC as \([^{11}\text{C}]61\). Interestingly, for \([^{11}\text{C}]61\) a similar metabolic profile was observed as for \([^{11}\text{C}]59\), the main difference being a slightly higher parent fraction at 45 minutes (45%).

15 min after injection of \([^{18}\text{F}]64\), 39% of the activity in the brain was due to parent compound. It should be noted that the fraction of radiolabeled non-polar metabolites in the brain was very low, whilst the fraction of polar metabolites was high. Most likely this is due to fast removal of the fluoroethyl moiety, which is capable of entering the brain [41].

![Figure 2. Fractions of radiolabeled polar metabolites, non-polar radiolabeled metabolites and parent compound of \([^{11}\text{C}]59\) (top), \([^{11}\text{C}]61\) (middle) and \([^{18}\text{F}]64\) (bottom) in plasma (left side) and brain (right side) at 15 and 45 min; n=3 for each data point; error bars indicate SD.](image-url)
3.2.6. Ex vivo biodistribution

Ex vivo biodistribution and regional brain uptake of $[^{11}C]59$, $[^{11}C]61$ and $[^{18}F]64$ were determined in male Wistar rats at 5, 15, 30 and 60 min after injection of the radiotracer. As anesthesia would interfere with binding to the NMDAr, these biodistribution studies were performed without anesthesia. Regional brain uptake as well as organ uptake are depicted in Figure 3.

Compared with all other time points, brain uptake of $[^{11}C]59$ was highest at 5 min after injection ($p < 0.05$, Tukey-test). Regionally, uptake was the lowest in cerebellum, and the highest ratio of radioactivity uptake between forebrain regions and cerebellum was observed at 15 min post injection. Two way ANOVA confirmed significant main effects of region [$F_{(4,60)} = 9.41, p < 0.0001$] and time [$F_{(3,60)} = 106.09, p < 0.0001$] on brain uptake of $[^{11}C]59$.

Organ uptake of $[^{11}C]59$ was highest in lungs followed by kidneys. Uptake in lungs was significantly higher than in other organs, except for kidney (15 min) and liver (30 min). The lowest levels of radioactivity were observed in blood and bone. Radioactivity cleared from most organs, but liver showed increasing uptake up to 45 min, which could be an indication of metabolism. Two way ANOVA confirmed significant main effects of region [$F_{(5,72)} = 72.78, p < 0.0001$] and time [$F_{(3,72)} = 14.12, p < 0.0001$], as well as significant organ × time interaction effects [$F_{(15,72)} = 6.33, p < 0.0001$].

Brain uptake of $[^{11}C]61$ was also highest at 5 min ($p < 0.001$, Tukey-test). In addition, similar to $[^{11}C]59$, uptake of $[^{11}C]61$ was lowest in cerebellum, and the highest ratio of radioactivity uptake between forebrain regions and cerebellum was found at 15 min post injection. Radioactivity cleared rapidly from the brain. Two way ANOVA confirmed significant main effects of region [$F_{(4,60)} = 24.11, p < 0.0001$] and time [$F_{(3,60)} = 696.02, p < 0.0001$] as well as significant region × time interaction effects [$F_{(12,60)} = 5.25, p < 0.0001$].

Organ uptake of $[^{11}C]61$ was highest in lungs followed by kidneys. The lowest level of radioactivity uptake was observed in blood. Remarkably, for a carbon-11 labeled tracer, uptake and washout over time was observed in bone. In addition, similar to the pattern seen for $[^{11}C]59$, the liver did not show a decrease of radioactivity levels over time. Two way ANOVA confirmed significant main effects of region [$F_{(5,72)} = 48.96, p < 0.0001$] and time [$F_{(3,72)} = 18.04, p < 0.0001$], as well as significant organ × time interaction effects [$F_{(15,72)} = 7.39, p < 0.0001$] on uptake of $[^{11}C]61$.

Uptake of $[^{18}F]64$ was uniformly distributed across the brain with a significant higher level of radioactivity at 15 min than at 30 min ($p < 0.05$, Tukey-test). Indeed, washout of radioactivity from the brain over time was not observed.
Figure 3. Biodistribution of $^{[11]C}59$, $^{[11]C}61$ and $^{[18]F}64$ in CNS (left) and organs (right) represented as %ID/g; n=4 for each data point; error bars indicate SD.

Organ uptake of $^{[18]F}64$ was highest in lungs followed by kidneys. At all time points, lung uptake was significantly higher than in other organs ($p < 0.001$). Relatively high
blood values were seen with increasing levels over time. The liver did not show a decrease over time and bone uptake was observed. These observations may be indicative of metabolism and in vivo defluorination of $[^{18}\text{F}]64$, although the latter is unlikely because bone uptake did not increase over time.

3.2.7. Pre-treatment studies

Specificity of uptake of the radiolabeled ligands $[^{11}\text{C}]59$, $[^{11}\text{C}]61$ and $[^{18}\text{F}]64$ in the brain was determined by pre-treatment with the NMDAr ion channel antagonist MK-801. Data from rodent studies had shown that 50% occupancy of the NMDAr ion-channel by MK-801 could be obtained using a dose of 0.18 mg·kg$^{-1}$ [42,43]. In the present study, 0.6 mg·kg$^{-1}$ MK-801 was used, which is at least three times the in vivo EC$_{50}$ value and therefore should occupy the majority of NMDArs. MK-801 was injected intraperitoneally 15 minutes prior to administration of the radiolabeled ligand.

No decrease in brain-uptake was observed for any of the three ligands (Figure 4). In fact, in all brain areas uptake of the radiolabeled ligands increased. For $[^{11}\text{C}]61$, this increase in uptake was significant (Two way ANOVA, Bonferroni posttests, P<0.01) in hippocampus, prefrontal cortex and cerebral cortex.

The reason for this higher uptake is unclear. One explanation could be that there is release of glutamate from the brain after MK-801 administration [44,45]. Since the uptake of ‘use-dependent’ antagonists is thought to be related to the amount of glutamate in the brain [46], it could be that injection of MK-801 increased the release of glutamate, opening more NMDAr ion channels, and resulting in higher uptake of $[^{11}\text{C}]59$, $[^{11}\text{C}]61$ and $[^{18}\text{F}]64$ than at baseline. In addition, it is possible that blood flow increased after the administration of MK-801, leading to increased delivery of the tested radioligands in the brain compared with the baseline condition [47].

3.3. Conclusion

Alkyl amine substitution of 5 and 6 at either methoxy or thiomethyl moieties led to three new ligands (59, 61 and 64) with favorable K$_i$ values towards the ion channel of the NMDAr. These ligands were radiolabeled and evaluated using ex vivo biodistribution studies in awake rats, showing good uptake in the brain. However, following pre-treatment with MK-801, none of the ligands tested showed reduced uptake. In addition, all three ligands suffered from fast metabolism. Despite the high affinity for the ion channel of the NMDAr, the radiolabeled ligands did not show appropriate characteristics for use as a PET tracer.
Figure 4. Control and pretreatment (15 minutes, MK-801 at 0.6 mg·kg⁻¹) biodistribution of \(^{[11C]}_59\), \(^{[11C]}_61\) and \(^{[18F]}_64\) in CNS and organs (represented as %ID/g (n=4 ± SD); **: P<0.01).
3.4. Experimental

3.4.1. Chemistry

Nuclear magnetic resonance (NMR) spectra (1H, 13C, 19F NMR) were recorded on a Bruker Avance 250 (250.13, 62.90, 235.36 MHz respectively). The chemical shifts of the NMR spectra are reported in parts per million (ppm) relative to the solvent residual peak. Description of signals: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, ddd = doublet of dd, dt = doublet of triplets, tt = triplet of triplets, dq = double quartet. Thin-layer chromatography (TLC) was performed on Merck DC-alufolien, silica gel 60, F254. Flash column chromatography was performed on silica gel 60Å, 230 – 400 Mesh. All solids obtained were dried *in vacuo*. All chemicals were used without further purification, unless stated otherwise. The HPLC analysis system consisted of a Jasco PU-2089 HPLC pump, a Rheodyne injector with a 20-μL loop, a Jasco UV-2075 Plus UV detector set at a wavelength of 254 nm and a Raytest Na(I) radioactivity detector. The HPLC data were collected and integrated with the software package GINA 5.01. HPLC: System A: Platinum EPS, C18 100Å 5µ, 250 x 4.6 mm column using 50 mM NH4H2PO4 pH=2.5 / methanol (30/70, v/v) as eluent and a flow rate of 1 mL·min⁻¹; For high-resolution mass spectrometry, a Finnigan MAT 90 was used. [11C]CO₂ was produced by the 14N(p,α)11C nuclear reaction and [18F]F⁻ was produced by the 18O(p,n)18F nuclear reaction with an IBA Cyclone 18/9 cyclotron. Radioactivity amounts were measured with a Veenstra VDC-405 dose calibrator. Radiochemistry was carried out in homemade, remotely controlled devices [48].

3.4.2. General Procedure for the synthesis of the di- or tri-N-Substituted Guanidines (A)

In a screw cap reaction vessel were dissolved the appropriately substituted cyanamide (1 mmol) and amine halogen salt (1.1 mmol) in chlorobenzene (200 μL). The reaction vessel was flushed with nitrogen, closed and stirred at 165 °C for 4 - 18 h. The reaction mixture was cooled down and dissolved in ethyl acetate (25 mL) and washed with 0.1M HCl (2 x 25 mL) followed by water (25 mL). The pH of the combined aqueous layers were adjusted with K₂CO₃ to pH ≥ 10 and extracted with ethyl acetate (2 x 25 mL). The organic layers were collected and dried over anhydrous magnesium sulfate, filtrated and evaporated to dryness under reduced pressure. The crude compound was purified by column chromatography. Oily products were converted into the corresponding fumaric acid salt by stirring the oil in diethyl ether.
and a solution of fumaric acid in diethyl ether was added dropwise. The resulting precipitate was filtered off.

3.4.2.1. N’-hydroxy-N-(pyridin-2-yl)formimidamide (43a)
To a stirred solution of 2-aminopyridine (42a) (5.48 g, 58.2 mmol) in 2-propanol (20 mL) DMF-DMA (10 mL, 75 mmol) was added. The reaction mixture was refluxed for 2 h under a nitrogen atmosphere and monitored by TLC (ethyl acetate). On completion, the reaction mixture was cooled to 50 °C and hydroxylamine hydrochloride (5.3 g, 76 mmol) was added and stirring was continued overnight. The solvents were evaporated under reduced pressure and the yellow syrup was treated with ethyl acetate (50 mL). The solids were filtered and the filtrate was washed with 1M NaOH (50 mL) and ethyl acetate (4 x 25 mL). The combined organic fractions were collected, dried over anhydrous magnesium sulfate, filtrated and evaporated to dryness under reduced pressure. The residue was recrystallized from chloroform to obtain the title compound as white crystals (4.62 g, 33.7 mmol, 58%). Rf 0.67 (ethyl acetate/methanol/Et3N, 90/10/1, v/v/v). 1H NMR (DMSO-d6) δ 10.06 (bs, 1H, OH), 9.30 (d, J=9.51Hz, 1H, NH), 8.14-8.11 (m, 1H, H Aryl), 7.87 (d, J=9.49Hz, 1H, CH), 7.61-7.54 (m, 1H, H Aryl), 7.05 (d, J=8.31Hz, 1H, H Aryl) , 6.84-6.79 (m, 1H, H Aryl). 13C NMR (DMSO-d6) δ 152.62 (Ar-N), 147.50 (Ar), 138.08 (Ar), 135.73 (CH), 116.29 (Ar), 110.40 (Ar).

3.4.2.2. N’-hydroxy-N-(pyridin-3-yl)formimidamide (43b)
To a stirred solution of 3-aminopyridine (42b) (5.47 g, 58.2 mmol) in 2-propanol (20 mL) DMF-DMA (10 mL, 75 mmol) was added. The reaction mixture was refluxed for 3 h under a nitrogen atmosphere and monitored by TLC (ethyl acetate/methanol 9/1, v/v). On completion, the reaction mixture was cooled to 75 °C and hydroxylamine hydrochloride (5.3 g, 76 mmol) was added and stirring was continued for 2 h. While evaporating under reduced pressure precipitation occurred. The yellow crystals were washed with 2-propanol and filtrated to obtain the title compound as light yellow crystals (4.18 g, 30.5 mmol, 52%). Rf 0.50 (ethyl acetate/methanol, 90/10, v/v). 1H NMR (DMSO-d6) δ 10.01 (bs, 1H, OH), 8.80 (d, J=10.60Hz, 1H, NH), 8.49 (d, J=2.53Hz, 1H, H Aryl), 8.06 (dd, J=4.60, 0.82Hz, 1H, H Aryl), 7.57-7.50 (m, 2H, H Aryl + CH), 7.21 (dd, J=8.30, 4.65Hz, 1H, H Aryl). 13C NMR (DMSO-d6) δ 141.42 (Ar), 137.52 (Ar-N), 137.40 (CH), 137.38 (Ar), 123.73 (Ar), 120.83 (Ar).

3.4.2.3. N’-hydroxy-N-(pyridin-4-yl)formimidamide (43c)
To a stirred solution of 4-aminopyridine (42c) (5.48 g, 58.2 mmol) in 2-propanol (20 mL) DMF-DMA (10 mL, 75 mmol) was added. The reaction mixture was refluxed for 2 h under a nitrogen atmosphere and monitored by TLC (ethyl acetate/methanol/Et3N 90/10/1, v/v/v). On completion, the reaction mixture was cooled to 50 °C and
hydroxylamine hydrochloride (5.3 g, 76 mmol) was added and stirring was continued for 3 h at 75 °C. While evaporating under reduced pressure precipitation occurred. The yellow crystals were washed with 2-propanol and filtrated to obtain the title compound as light tanned crystals (5.67 g, 41.3 mmol, 71%). Rf 0.33 (ethyl acetate/methanol/Et3N, 90/10/1, v/v/v). 1H NMR (DMSO-d6) δ 10.51 (bs, 1H, OH), 9.62 (d, J=10.03Hz, 1H, NH), 8.32 (d, J=6.59Hz, 2H, H Aryl), 7.71 (d, J=9.79Hz, 1H, CH), 7.31 (d, J=6.66Hz, 2H, H Aryl). 13C NMR (DMSO-d6) δ149.28 (Ar-N), 147.13 (2 x Ar), 135.79 (CH), 109.57 (2 x Ar).

3.4.2.4. N-methyl-N-(pyridin-2-yl)cyanamide (44a)
To a stirred solution of N'-hydroxy-N-(pyridin-2-yl)formimidamide (43a) (686 mg, 5.00 mmol) in toluene (13 mL) was added DMF-DMA (1.65 mL, 12.3 mmol) and refluxed for 2.5 h. The solvents were evaporated and the yellow residue solidified on standing which was triturated with toluene. The yellow needles were collected to obtain the title compound (198 mg, 1.49 mmol, 30%). Rf 0.28 (ethyl acetate). 1H NMR (CDCl3) δ 8.01 (dd, J=6.61Hz,1.35Hz, 1H, H Aryl), 7.76-7.69 (m, 1H, H Aryl), 7.12 (d, J=8.76Hz, 1H, H Aryl), 6.7 (dt, J=6.77Hz,1.25Hz, 1H, H Aryl), 3.61 (s, 3H, CH3). 13C NMR (CDCl3) δ 156.22 (Ar-N), 134.08 (Ar), 130.57 (Ar), 129.18 (Ar), 126.95 (Ar), 121.92 (NCN), 47.06 (CH3).

3.4.2.5. N-methyl-N-(pyridin-3-yl)cyanamide (44b)
To a stirred solution of N'-hydroxy-N-(pyridin-3-yl)formimidamide (43b) (1.37 g, 10.0 mmol) in toluene (25 mL) was added DMF-DMA (3.3 mL, 25 mmol) and refluxed for 35 minutes. On cooling crystals were formed. After filtering, the title compound was obtained as brown solid (426 mg, 3.20 mmol, 32%). Rf 0.18 (ethyl acetate/methanol, 90/10, v/v). 1H NMR (DMSO-d6) δ 7.91 (s, 1H, H Aryl), 7.79 (d, J=5.06Hz, 1H, H Aryl), 7.53-7.42 (m, 2H, H Aryl), 4.11 (s, 3H, CH3). 13C NMR (DMSO-d6) δ 156.22 (Ar-N), 134.08 (Ar), 130.57 (Ar), 129.18 (Ar), 126.95 (Ar), 121.92 (NCN), 47.06 (NCH3).

3.4.2.6. N-methyl-N-(pyridin-4-yl)cyanamide (44c)
To a stirred solution of N'-hydroxy-N-(pyridin-4-yl)formimidamide (43c) (1.37 g, 10.0 mmol) in toluene (25 mL) was added DMF-DMA (3.3 mL, 25 mmol) and refluxed for 30 minutes. On cooling white crystals were formed. After filtering, the title compound was obtained as white needles (809 mg, 6.08 mmol, 61%). Rf 0.15 (ethyl acetate/methanol/Et3N, 90/10/1, v/v/v). 1H NMR (DMSO-d6) δ 7.88 (d, J=7.32Hz, 2H, H Aryl), 6.69 (bs, 2H, H Aryl), 3.78 (s, 3H, NCH3). 13C NMR (DMSO-d6) δ 167.28 (Ar-N), 142.13 (2 x Ar), 119.71 (NCN), 114.40 (2 x Ar), 43.68 (NCH3).
3.4.2.7. 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-(pyridin-2-yl)guanidine (45a)

The reaction of N-methyl-N-(pyridin-2-yl)cyanamide (44a) (135 mg, 1.01 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (233 mg, 1.11 mmol) according to procedure A afforded, after purification over silica with methanol/chloroform/Et$_3$N (50/50/1, v/v/v), the title compound as a light yellow glassy solid (105 mg, 0.34 mmol, 34%). $R_f$ 0.09 (methanol/chloroform/Et$_3$N, 50/50/1, v/v/v). $^1$H NMR (CDCl$_3$) δ 7.79 (bs, 1H, H Aryl), 7.30-7.16 (m, 3H, H Aryl), 6.97 (d, J=2.26Hz, 1H, H Aryl), 6.79 (dd, J=8.35, 2.28Hz, 1H, H Aryl), 6.08 (dt, J=6.66, 1.19Hz, 1H, H Aryl), 4.71 (bs, 2H, NH), 3.53 (s, 3H, NCH$_3$), 2.42 (s, 3H, NCH$_3$). $^{13}$C NMR (CDCl$_3$) δ 179.29 (Ar-NCH$_3$), 156.87 (NCN), 156.09 (Ar-NH), 138.28 (Ar), 137.24 (Ar-S), 137.04 (Ar), 129.79 (Ar), 124.44 (Ar-Cl), 122.26 (Ar), 121.16 (Ar), 118.10 (Ar), 106.62 (Ar), 40.46 (NCH$_3$), 15.97 (SCH$_3$). The free base was converted into its fumaric acid salt (116 mg, 0.31 mmol, 92%). $^1$H NMR (DMSO-d$_6$) δ 7.88 (d, J=6.47Hz, 1H, H Aryl), 7.56-7.49 (m, 1H, H Aryl), 7.41 (bs, 2H, NH), 7.30 (d, J=8.40Hz, 1H, H Aryl), 7.16 (d, 8.77Hz, 1H, H Aryl), 7.07 (d, J=2.08Hz, 1H, H Aryl), 6.91 (dd, J=8.41, 2.23Hz, 1H, H Aryl), 6.48 (t, J=6.57Hz, 1H, H Aryl), 6.35 (s, 0.88H, fumaric acid), 3.48 (s, 3H, NCH$_3$), 2.39 (s, 3H, SCH$_3$). HRMS [M + H]$^+$ calcd for C$_{14}$H$_{15}$ClN$_4$S 307.0779, found 307.0778. HPLC system A: purity 99.2%, rt = 6.22 min.

3.4.2.8. 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-(pyridin-4-yl)guanidine (45c)

The reaction of N-methyl-N-(pyridin-4-yl)cyanamide (44c) (134 mg, 1.01 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (232 mg, 1.10 mmol) according to procedure A afforded, after recrystallization from chloroform / hexanes the title compound as light brown crystals (121 mg, 0.39 mmol, 39%). $R_f$ 0.08 (methanol/chloroform/Et$_3$N, 50/50/1, v/v/v). $^1$H NMR (DMSO-d$_6$) δ 7.41 (d, J=7.59Hz, 2H, H Aryl), 7.17 (d, J=8.29Hz, 1H, H Aryl), 6.74 (d, J=2.28Hz, 1H, H Aryl), 6.64 (dd, J=8.30, 2.34Hz, 1H, H Aryl), 6.49 (d, J=7.12Hz, 2H, H Aryl), 5.63 (bs, 2H, NH), 3.53 (s, 3H, NCH$_3$), 2.34 (s, 3H, SCH$_3$). $^{13}$C NMR (DMSO-d$_6$) δ 158.43 (Ar-NCH$_3$), 157.68 (NCN), 148.42 (Ar-NH), 139.65 (Ar), 136.05 (Ar-S), 129.13 (Ar), 123.33 (Ar-Cl), 121.42 (Ar), 118.84 (Ar), 112.38 (Ar), 42.39 (NCH$_3$), 14.99 (SCH$_3$). HRMS [M + H]$^+$ calcd for C$_{14}$H$_{15}$ClN$_4$S 307.0779, found 307.0779. HPLC system A: purity 99.0%, rt = 6.22 min.

3.4.2.9. 2,5-dimethyl-1-(3-nitrophenyl)-1H-pyrrole (47)

In a Dean-Stark setup a solution of 3-nitroaniline (46) (13.9 g, 100 mmol), a catalytic amount of p-toluenesulfonic acid monohydrate (0.29g, 1.5 mmol) in toluene (350 mL) was stirred at elevated temperature. After 5 minutes hexane-2,5-dione (12.5 mL, 107 mmol) was added via an addition funnel and refluxed under nitrogen atmosphere for 75 min. After cooling the reaction was quenched with sat. aq. NaHCO$_3$ solution (100 mL) and separated. The organic layer was washed with water (100 mL), 0.1M NaOH (2 x 50 mL), 0.1M HCl (2 x 50 mL), water (50 mL) and brine (50 mL). The organic
fraction was collected, dried over anhydrous magnesium sulfate, filtrated and evaporated to dryness under reduced pressure. On cooling the brown oil crystallized to obtain the title compound (21.7 g, 100 mmol, 100%). \( R_f \) 0.84 (ethyl acetate/hexanes, 33/66, v/v). \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 8.28 (ddd, \( J=8.12Hz, 2.22Hz, 1.21Hz, \) 1H, HAryl), 8.12 (t, \( J=2.01Hz, 1H, \) H\textsubscript{Aryl}), 7.68 (t, \( J=7.92Hz, 1H, \) H\textsubscript{Aryl}), 7.60-7.56 (m, 1H, H\textsubscript{Aryl}), 5.95 (s, 2H, pyrrole), 2.07 (s, 6H, CH\(_3\)). \( ^{13}C \) NMR (CDCl\(_3\)) \( \delta \) 148.80 (Ar-NO\(_2\)), 140.35 (Ar-pyrrole), 134.38 (Ar), 130.07 (Ar), 128.75 (pyrrole-CH\(_3\)), 123.39 (Ar), 122.62 (Ar), 107.11 (pyrrole-CH)13.15 (CH\(_3\)).

3.4.2.10.3-(2,5-dimethyl-1H-pyrrol-1-yl)aniline (48)

To a refluxing solution of 2,5-dimethyl-1-(3-nitrophenyl)-1H-pyrrole (47) (5.00g, 23.1 mmol) and acetic acid (6.5 mL, 114 mmol) in ethanol / water (19 5 mL, 2/1, v/v) was added powdered iron (5.2 g, 93 mmol) portion wise. The reaction mixture was refluxed for 18 minutes, when TLC showed completion. After cooling the mixture was basified with 25% NH\(_4\)OH and filtered through celite. The filter cake was washed with ethanol and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography over silica with dichloromethane to obtain the title compound as a light brown solid (6.97 g, 21.3 mmol, 92%). \( R_f \) 0.48 (ethyl acetate/hexanes, 25/75, v/v). \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 7.24 (t, \( J=7.94Hz, 1H, \) H\textsubscript{Aryl}), 6.72 (ddd, 1H, H\textsubscript{Aryl} J=8.06, 2.30, 0.92Hz), 6.62 (ddd, 1H, H\textsubscript{Aryl} J=7.73, 1.85, 0.94), 6.53 (t, 1H, H\textsubscript{Aryl}, J=2.06Hz), 5.91 (s, 2H, CH-pyrrole), 3.78 (bs, 2H, NH\(_2\)), 2.08 (s, 6H, CH\(_3\)). \( ^{13}C \) NMR (CDCl\(_3\)) \( \delta \) 147.21 (Ar-NH\(_2\)), 140.13 (Ar-pyrrole), 129.81 (Ar), 128.85 (C-pyrrole), 118.49 (Ar), 114.91 (Ar), 114.40 (Ar), 105.49 (CH-pyrrole), 13.05 (CH\(_3\)).

3.4.2.11.N-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)cyanamide (49)

To a stirred mixture of 3-(2,5-dimethyl-1H-pyrrol-1-yl)aniline (48) (3.88 g, 20.8 mmol) and anhydrous sodium acetate (5.19 g, 63.3 mmol) in methanol (35 mL) at 0 ° was added a solution of cyanogen bromide [CAUTION highly toxic] (2.88 g, 27.2 mmol) in methanol (35 mL) via an addition funnel. After 2 h of stirring the ice-bath was removed and stirring was continued for 24 h. The reaction mixture was diluted with dichloromethane (150 mL) and washed with 1M HCl (150 mL). The aqueous layer was washed with dichloromethane (150 mL). The combined organic layer was washed with brine (75 mL), dried over anhydrous magnesium sulfate, filtered and evaporated to dryness. The crude product was purified with flash column chromatography (dichloromethane) to obtain the title compound as light brown solid (3.35 g, 15.9 mmol, 76%). \( R_f \) 0.37 (ethyl acetate/hexanes, 25/75, v/v). \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 7.44 (t, \( J=8.01Hz, 1H, \) H\textsubscript{Aryl}), 7.08-7.04 (m, 1H, H\textsubscript{Aryl}), 6.98-6.94 (m, 1H, H\textsubscript{Aryl}), 6.86 (t, \( J=2.04Hz, 1H, \) H\textsubscript{Aryl}), 5.91 (s, 2H, CH-pyrrole), 2.04 (s, 6H, CH\(_3\)). \( ^{13}C \) NMR (CDCl\(_3\)) \( \delta \)
3.4.2.12. N-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-N-methylcyanamide (50)

To a stirred solution of N-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)cyanamide (49) (3.27 g, 15.5 mmol) in dry DMF (60 mL) was added potassium carbonate (2.58 g, 18.6 mmol). After 5 minutes of stirring methyl iodide (3 mL, 48 mmol) was added and stirring was continued for 1.5 h. The solvent was evaporated under reduced pressure and the residue was dissolved in water (125 mL) and extracted with ethyl acetate (2 x 125 mL). The combined organic layer was washed with brine (100 mL), collected and dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure. The crude product was purified with flash column chromatography (ethyl acetate/hexanes (25/75, v/v)) to obtain the title compound as a light brown solid (3.22 g, 14.3 mmol, 92%). Rf 0.56 (ethyl acetate/hexanes, 25/75, v/v). 1H NMR (CDCl3) δ 7.48 (t, J=8.04Hz, 1H, H Aryl), 7.19-7.14 (m, 1H, H Aryl), 6.99-6.92 (m, 2H, H Aryl), 5.91 (s, 2H, CH-pyrrole), 3.36 (s, 3H, NCH3), 2.04 (s, 6H, CH3). 13C NMR (CDCl3) δ 141.33 (Ar-NCH3), 140.40 (Ar-pyrrole), 130.24 (Ar), 128.64 (C-pyrrole), 123.11 (Ar), 114.67 (Ar), 114.17 (Ar), 113.50 (NCN), 106.15 (CH-pyrrole), 36.93 (NCH3), 13.00 (CH3).

3.4.2.13. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-1-methylguanidine (51)

The reaction of N-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-N-methylcyanamide (50) (698 mg, 3.10 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (717 mg, 3.41 mmol) according to procedure A afforded, after purification over silica with ethyl acetate/hexanes (25/75 to 33/66, v/v), the title compound as a colorless oil (569 mg, 1.43 mmol, 46%). Rf 0.36 (dichloromethane/methanol, 50/50, v/v). 1H NMR (CDCl3) δ 7.51 (t, J=7.88Hz, 1H, H Aryl), 7.40 (m, 1H, H Aryl), 7.29 (d, J=8.33Hz, 1H, H Aryl), 7.23 (t, J=1.96Hz, 1H, H Aryl), 7.12 (m, 1H, H Aryl), 6.93 (d, J=2.88Hz, 1H, H Aryl), 6.84 (dd, J=8.34, 2.32Hz, 1H, H Aryl), 5.93 (s, 2H, CH-pyrrole), 4.01 (bs, 2H, NH), 3.47 (s, 3H, NCH3), 2.48 (s, 3H, SCH3), 2.09 (s, 6H, CH3). 13C NMR (CDCl3) δ 150.65 (Ar-NCH3), 147.20 (NCN), 145.35 (Ar-NH), 140.09 (Ar-pyrrole), 137.83 (Ar-S), 130.08 (Ar), 130.05 (Ar), 128.52 (C-pyrrole), 126.28 (Ar), 125.81 (Ar), 125.44 (Ar), 124.25 (Ar-Cl), 122.28 (Ar), 121.42 (Ar), 106.26 (CH-pyrrole), 38.90 (NCH3), 15.93 (SCH3), 13.14 (CH3).

3.4.2.14. 1-(3-aminophenyl)-3-(2-chloro-5-(methylthio)phenyl)-1-methylguanidine (52)

To a solution of hydroxylamine hydrochloride (497 mg, 7.15 mmol) in ethanol / water (5.25 mL, 11/4, v/v) was added 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-1-methylguanidine (51) (569 mg, 1.43 mmol). After
refluxing for 24 h the reaction mixture was poured into 2M HCl (50 mL) and washed twice with diethyl ether (25 mL). The aqueous layer was cooled in an ice-bath and basified with solid sodium hydroxide and extracted twice with diethyl ether (25 mL). The combined organic fraction was dried over anhydrous magnesium sulfate, filtered and evaporated to dryness. The crude product was purified with flash column chromatography (ethyl acetate/Et3N, 99/1, v/v) to obtain the title compound as light yellow oil (451 mg, 1.41 mmol, 99%). Rf 0.28 (ethyl acetate/Et3N, 99/1, v/v). 1H NMR (CDCl3) δ 7.21 (d, J=8.33Hz, 1H, H_Aryl), 7.06 (t, J=7.91Hz, 1H, H_Aryl), 6.85 (d, J=2.29Hz, 1H, H_Aryl), 6.74 (dd, J=8.35, 2.33Hz, 1H, H_Aryl), 6.61-6.57 (m, 1H, H_Aryl), 6.51 (t, J=2.04Hz, 1H, H_Aryl), 6.47-6.42 (m, 1H, H_Aryl), 3.39 (bs, 2H, NH), 3.83 (bs, 2H, NH2), 3.29 (s, 3H, NCH3), 2.37 (s, 3H, SCH3). 13C NMR (CDCl3) δ 150.92 (Ar-NH2), 147.86 (Ar-NCH3), 147.58 (NCN), 145.10 (Ar-NH), 137.45 (Ar-S), 130.23 (Ar), 129.85 (Ar), 124.43 (Ar-Cl), 122.40 (Ar), 116.24 (Ar), 113.21 (Ar), 38.41 (NCH3), 15.70 (SCH3). The free base (100 mg) was converted into its fumaric acid salt (119 mg, 0.27 mmol, 87%). 1H NMR (DMSO-d6) δ 7.30 (d, J=8.02Hz, 1H, H_Aryl), 7.02 (t, J=7.86Hz, 1H, H_Aryl), 6.90-6.85 (m, 2H, H_Aryl), 6.55 (s, 1.83Hz, fumaric acid), 6.49-6.40 (m, 3H, H_Aryl), 3.24 (s, 3H, NCH3), 2.44 (s, 3H, SCH3). HRMS [M + 2Na – H] calcd for C15H17ClN4S 366.0647, found 365.1215. HPLC system A: purity 99.2%, rt = 4.17 min.

3.4.2.15. N-(3-nitrophenyl)cyanamide (53)
To a cooled suspension (0 °C) of 3-nitroaniline (46) (13.8g, 100 mmol) in acetic acid / water (188 mL, 3/1, v/v) was added cyanic bromide [CAUTION highly toxic] (16.0g, 151 mmol). After 20 minutes 1M NaOH (150 mL) was added over a period of 10 min, the reaction was warmed to room temperature and stirred for 20 h. The suspension was filtrated and the filter cake was washed with ethanol (1st crop). The filtrate was evaporated under reduced pressure and dissolved in 1M NaOH (100 mL), 1M HCl was added until precipitation occurred. The crystals were collected (2nd crop). Both crops were combined and the title compound was obtained as light gray crystals (15.1 g, 92.5 mmol, 93%). Rf 0.80 (ethyl acetate/hexanes/Et3N, 50/50/1, v/v/v). 1H NMR (DMSO-d6) δ 10.86 (bs, 1H, NH), 7.90-7.86 (m, 1H, H_Aryl), 7.71-7.61 (m, 2H, H_Aryl), 7.42-7.38 (m, 1H, H_Aryl). 13C NMR (DMSO-d6) δ 148.57 (Ar-NO2), 140.28 (Ar-NH), 131.31 (Ar), 121.33 (Ar), 117.22 (Ar), 111.03 (NCN), 109.36 (Ar).

3.4.2.16. N-methyl-N-(3-nitrophenyl)cyanamide (54)
To a mixture of N-(3-nitrophenyl)cyanamide (53) (12.3 g, 75.5 mmol) and potassium carbonate (13 g, 94 mmol) in DMF (76 mL) was added methyl iodide (7.1 mL, 114 mmol) and stirred for 45 minutes at 45 °C. The solvent was evaporated under reduced pressure and the residue was dissolved in water (150 mL) and extracted twice with ethyl acetate (125 mL). The combined organic layer was washed with brine (100mL),
collected, dried over anhydrous magnesium sulfate, filtrated and evaporated to dryness under vacuo. The crude product was purified by flash column chromatography (dichloromethane) to obtain the title compound as a light yellow solid (12.1 g, 68.2 mmol, 90%). Rf 0.62 (dichloromethane). 1H NMR (CDCl3) δ 8.00-7.96 (m, 1H, H_Aryl), 7.87 (t, J=4.18Hz, 1H, H_Aryl), 7.58 (t, J=8.09Hz, 1H, H_Aryl), 7.52-7.47 (m, 1H, H_Aryl), 3.45 (s, 3H, NCH3). 13C NMR (CDCl3) δ 149.11 (Ar-NO2), 141.83 (Ar-NH), 130.70 (Ar), 120.93 (Ar), 118.12 (Ar), 112.69 (NCN), 109.32 (Ar), 37.16 (NCH3).

3.4.2.17. N-(3-aminophenyl)-N-methylcyanamide (55)

To a refluxing solution of N-methyl-N-(3-nitrophenyl)cyanamide (54) (10.6 g, 60.0 mmol) and acetic acid (17.3 mL, 302 mmol) in ethanol / water (600 mL, 2/1, v/v) was added powdered iron (13.5 g, 241 mmol) in portions. After 10 minutes TLC showed complete conversion and the reaction was cooled to room temperature. The reaction mixture was basified with NH4OH (25%) and filtered through celite. The filter cake was washed with methanol. The filtrate was concentrated under reduced pressure and crystallized from methanol / water to obtain the title compound as brown needles (7.60 g, 51.6 mmol, 86%). Rf 0.63 (dichloromethane/methanol, 95/5, v/v). 1H NMR (CDCl3) δ 7.13 (t, J=8.02Hz, 1H, H_Aryl), 6.43-6.40 (m, 3H, H_Aryl), 3.79 (bs, 2H, NH2), 3.29 (s, 3H, NCH3). 13C NMR (CDCl3) δ 148.14 (Ar-NH2), 140.75 (Ar-N), 129.65 (Ar), 113.82 (NCN), 109.40 (Ar), 103.10 (Ar), 100.48 (Ar), 36.06 (NCH3).

3.4.2.18. 2,2,2-trifluoro-N-(3-(N-methylcyanamido)phenyl)acetamide (56)

To a suspension of N-(3-aminophenyl)-N-methylcyanamide (55) (1.47 g, 10.0 mmol) in dichloromethane (25 mL) at 0 °C was added trifluoroacetic anhydride (4.3 mL, 30 mmol). The reaction mixture was stirred for 1 h at 0 °C and the solvent was evaporated under reduced pressure. The title compound was obtained after repeated crystallizations from acetonitrile / water (2.08 g, 8.56 mmol, 86%). Rf 0.34 (dichloromethane). 1H NMR (CDCl3) δ 10.61 (bs, 1H, NH), 7.30-7.24 (m, 2H, H_Aryl), 7.06 (t, J=8.14Hz, 1H, H_Aryl), 6.60 (dd, J=7.96, 1.20Hz, H_Aryl), 3.05 (s, 3H, NCH3). 13C NMR (CDCl3) δ 154.17 (d, J=37.93Hz, CO), 140.42 (Ar-NC), 137.57 (Ar-NHCO), 129.49 (Ar), 115.30 (d, J=288.38Hz, CF3), 114.98 (Ar), 113.17 (NCN), 110.97 (Ar), 106.91 (Ar), 36.22 (NCH3).

3.4.2.19. 2,2,2-trifluoro-N-methyl-N-(3-(N-methylcyanamido)phenyl)acetamide (57)

To a stirred suspension of 2,2,2-trifluoro-N-(3-(N-methylcyanamido)phenyl)acetamide (56) (1.86 g, 7.64 mmol) and potassium carbonate (1.70 g, 8.46 mmol) in dry DMF (15 mL) was added methyl iodide (1 mL, 16.0 mmol). Stirring was continued at 45 °C for 2.5 h before filtering over celite. The filtrate was evaporated to dryness under reduced pressure. The crude product was purified over silica (dichloromethane) to obtain the title compound as a white solid (1.91 g, 7.41 mmol,
97%). \(R_f\) 0.55 (dichloromethane). \(^1H\) NMR (CDCl\(_3\)) \(\delta\) 7.42 (t, \(J=8.36\) Hz, 1H, H\(_{\text{Aryl}}\)), 7.13-7.09 (m, 1H, H\(_{\text{Aryl}}\)), 6.98-6.95 (m, 2H, H\(_{\text{Aryl}}\)). 3.32 (s, 3H, N(CH\(_3\))CO), 3.31 (s, 3H, NCH\(_3\)).

\(^13C\) NMR (CDCl\(_3\)) \(\delta\) 156.60 (d, \(J=38.71\) Hz, CO), 141.93 (Ar-N(CH\(_3\))CO), 141.67 (Ar-NCN), 130.78 (Ar), 122.12 (Ar), 118.52 (d, \(J=288.37\) Hz, CF\(_3\)), 115.30 (Ar), 113.89 (Ar), 113.18 (NCN), 39.57 (N(CH\(_3\))CO), 36.87 (NCH\(_3\)).

3.4.2.20. \(N\)-(3-(dimethylamino)phenyl)-N-methylcyanamide (58)

To a suspension of \(N\)-(3-aminophenyl)-N-methylcyanamide (55) (295 mg, 2.00 mmol) and potassium carbonate (683 mg, 5.01 mmol) in dry DMF was added methyl iodide (375 µL, 6.00 mmol) and stirred at 45 °C for 1 h. The solvent was evaporated under reduced pressure and the residue was dissolved in water (25 mL) and extracted twice with ethyl acetate (25 mL). The combined organic fraction was washed with brine (20 mL), collected, dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure. The crude product was purified with flash column chromatography (dichloromethane) to obtain the title compound as light yellow oil (179 mg, 1.02 mmol, 51%). \(R_f\) 0.60 (dichloromethane). \(^1H\) NMR (CDCl\(_3\)) \(\delta\) 7.19 (t, \(J=8.08\) Hz, 1H, H\(_{\text{Aryl}}\)), 6.46-6.34 (m, 3H, H\(_{\text{Aryl}}\)). 3.28 (s, 3H, CH\(_3\)), 2.96 (s, 6H, N(CH\(_3\))\(_2\)). \(^13C\) NMR (CDCl\(_3\)) \(\delta\) 151.39 (Ar-N(CH\(_3\))\(_2\)), 141.32 (Ar-N), 129.95 (Ar), 114.51 (NCN), 107.58 (Ar), 102.39 (Ar), 98.76 (Ar), 40.30 (N(CH\(_3\))\(_2\)), 36.73 (NCH\(_3\)).

3.4.2.21. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(dimethylamino)phenyl)-1-methylguanidine (59)

The reaction of \(N\)-(3-(dimethylamino)phenyl)-N-methylcyanamide (58) (176 mg, 1.00 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (232 mg, 1.10 mmol) in chlorobenzene (200 µL) according to procedure A afforded, after purification over silica with a gradient of ethyl acetate/hexanes/Et\(_3\)N (20/80/1 to 33/66/1, v/v/v), the title compound as a light yellow oil (206 mg, 0.59 mmol, 59%). \(R_f\) 0.19 (ethyl acetate/hexanes/Et\(_3\)N, 33/66/1, v/v/v). \(^1H\) NMR (CDCl\(_3\)) \(\delta\) 7.30-7.22 (m, 2H, H\(_{\text{Aryl}}\)), 6.95 (d, \(J=2.16\) Hz, 1H, H\(_{\text{Aryl}}\)), 6.81 (dd, \(J=8.33\) Hz, \(J=2.21\) Hz, 1H, H\(_{\text{Aryl}}\)). 6.68-6.63 (m, 3H, H\(_{\text{Aryl}}\)), 3.98 (bs, 2H, NH), 3.43 (s, 3H, NCH\(_3\)), 2.99 (s, 6H, N(CH\(_3\))\(_2\)), 2.47 (s, 3H, SCH\(_3\)). \(^13C\) NMR (CDCl\(_3\)) \(\delta\) 151.55 & 151.09 (Ar-NCH\(_3\) & Ar-N(CH\(_3\))\(_2\)), 147.72 (NCN), 145.15 (Ar-NH), 137.49 (Ar-S), 130.12 (Ar), 129.92 (Ar), 124.55 (Ar-Cl), 122.51 (Ar), 120.91 (Ar), 114.57 (Ar), 110.81 (Ar), 110.79 (Ar), 40.38 (N(CH\(_3\))\(_2\)), 38.66 (NCH\(_3\)), 15.86 (SCH\(_3\)). The free base was converted into its fumaric acid salt (176 mg, 0.39 mmol, 66%). \(^1H\) NMR (DMSO-\(d_6\)) \(\delta\) 7.31 (d, \(J=8.15\) Hz, 1H, H\(_{\text{Aryl}}\)), 7.19 (t, \(J=8.17\) Hz, 1H, H\(_{\text{Aryl}}\)), 6.90-6.84 (m, 2H, H\(_{\text{Aryl}}\)), 6.62-6.56 (m, 3H, H\(_{\text{Aryl}}\)), 6.55 (s, 1H, fumaric acid), 5.99 (bs, 2H, NH), 3.30 (s, 3H, NCH\(_3\)), 2.91 (s, 6H, N(CH\(_3\))\(_2\)), 2.44 (s, 3H, SCH\(_3\)). HRMS \([M + H]^+\) calcld for C\(_{17}\)H\(_{21}\)ClFN\(_4\)S 349.1248, found 349.1262. HPLC system A: purity 95.9%, rt = 5.47 min.
3.4.2.22. \( N-(3-(2-chloro-5-(methylthio)phenyl)-1-methylguanidino)phenyl)-2,2,2-trifluoro-N-methylacetamide (60) \)

The reaction of 2,2,2-trifluoro-\( N \)-methyl-\( N \)-(3-(N-methylcyanamido)phenyl)acetamide (57) (257 mg, 1.00 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (231 mg, 1.01 mmol) according to procedure A afforded, after purification over silica with ethyl acetate/hexanes/Et\(_3\)N (66/33/1, v/v/v), the title compound as a light yellow oil (177 mg, 0.41 mmol, 41%). \( R_f \) 0.21 (ethyl acetate/hexanes/Et\(_3\)N, 66/33/1, v/v/v). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 7.50-7.38 (m, 2H, H Aryl), 7.28-7.25 (m, 2H, H Aryl), 7.14-7.11 (m, 1H, H Aryl), 6.89 (d, \( J = 2.25 \) Hz, 1H, H Aryl), 6.82 (dd, \( J = 8.34, 2.38 \) Hz, 1H, H Aryl), 4.05 (bs, 2H, NH), 3.42 (s, 3H, NCH\(_3\)), 3.36 (s, 3H, N(CO)(CH\(_3\))), 2.45 (s, 3H, SCH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \) 156.33 (q, \( J_{CF} = 35.71 \) Hz, CO), 150.49 (Ar-N(CO)), 146.94 (Ar-NCH\(_3\)), 145.61 (NCN), 141.24 (Ar-NH), 137.70 (Ar-S), 130.47 (Ar), 129.91 (Ar), 126.68 (Ar), 125.32 (Ar), 124.62 (Ar), 123.99 (Ar-Cl), 121.99 (Ar), 121.14 (Ar), 116.18 (d, \( J_{CF} = 288.34 \) Hz, CF\(_3\)), 39.39 (CON(CH\(_3\))), 38.60 (NCH\(_3\)), 15.66 (SCH\(_3\)). \(^{19}\)F NMR (CDCl\(_3\)) \( \delta \) -66.88 (s, CF\(_3\)).

3.4.2.23. \( 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-(3-(methylamino)phenyl)guanidine (61) \)

To a solution of \( N-(3-(3-(2-chloro-5-(methylthio)phenyl)-1-methylguanidino)phenyl)-2,2,2-trifluoro-N-methylacetamide \) (60) (177 mg, 0.41 mmol) in methanol/water (4 mL, 5/1, v/v) was added potassium carbonate (52 mg, 0.38 mmol) and stirred at room temperature for 1h. The reaction mixture was diluted with water (25 mL) and extracted twice with ethyl acetate (25 mL). The combined organic fraction was washed with brine (25 mL), dried over anhydrous magnesium sulfate, filtered and evaporated under reduced pressure. The crude product was purified over silica (gradient dichloromethane to ethyl acetate/hexanes/Et\(_3\)N, 33/66/1, v/v/v) to obtain the title compound as a colorless oil (135 mg, 0.40 mmol, 98%). \( R_f \) 0.12 (ethyl acetate/hexanes/Et\(_3\)N, 33/66/1, v/v/v). \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 7.30 (d, \( J = 8.34 \) Hz, 1H, H Aryl), 7.19 (t, \( J = 7.90 \) Hz, 1H, H Aryl), 6.95 (d, \( J = 2.26 \) Hz, 1H, H Aryl), 6.82 (dd, \( J = 8.35, 2.31 \) Hz, 1H, H Aryl), 6.65-6.61 (m, 1H, H Aryl), 6.53-6.47 (m, 2H, H Aryl), 4.11 (bs, 1H, NH(CH\(_3\))), 4.01 (bs, 2H, NH), 3.41 (s, 3H, NCH\(_3\)), 2.80 (s, 3H, NH(CH\(_3\))), 2.46 (s, 3H, SCH\(_3\)). \(^{13}\)C NMR (CDCl\(_3\)) \( \delta \) 150.94 (Ar-NHCH\(_3\)), 150.46 (Ar-NCH\(_3\)), 147.56 (NCN), 145.02 (Ar-NH), 137.38 (Ar-S), 130.07 (Ar), 129.79 (Ar), 124.38 (Ar-Cl), 121.99 (Ar), 121.14 (Ar), 116.18 (d, \( J_{CF} = 288.34 \) Hz, CF\(_3\)), 39.39 (CON(CH\(_3\))), 38.60 (NCH\(_3\)), 30.32 (NCH\(_3\)), 15.63 (SCH\(_3\)). The free base was converted into its fumaric acid salt (138 mg, 0.30 mmol, 75%). \(^1\)H NMR (DMSO-\( d_6 \)) \( \delta \) 7.30 (d, \( J = 8.16 \) Hz, 1H, H Aryl), 7.12-7.05 (m, 1H, H Aryl), 6.90-6.86 (m, 2H, H Aryl), 6.55 (s, 2H, fumaric acid), 6.47-6.40 (m, 3H, H Aryl), 6.09 (bs, 3H, NH), 3.26 (s, 3H, NCH\(_3\)), 2.66 (s, 3H, NH(CH\(_3\))), 2.44 (s, 3H, SCH\(_3\)). HRMS [M + H]\(^+\) calcd for C\(_{16}\)H\(_{19}\)ClN\(_4\)S 335.1092, found 335.1097. HPLC system A: purity 97.9%, \( rt = 4.15 \) min.
3.4.2.24. N-methyl-N-(3-(methylamino)phenyl)cyanamide (62)

To a solution of 2,2,2-trifluoro-N-methyl-N-(3-(N-methylcyanamido)phenyl)acetamide (57) (1.29 g, 5.01 mmol) in methanol/water (50 mL, 5/1, v/v) was added potassium carbonate (0.69 g, 5.01 mmol) and stirred at room temperature for 1 h. The reaction mixture was diluted with water (200 mL) and extracted twice with ethyl acetate (50 mL). The combined organic fraction was washed with brine (25 mL), dried over anhydrous magnesium sulfate, filtered and evaporated under reduced pressure. The crude product was purified over silica (gradient dichloromethane to ethyl acetate/hexanes/Et₃N, 33/66/1, v/v/v) to obtain the title compound as an off-white solid (0.79 g, 4.88 mmol, 97%). R_f 0.44 (ethyl acetate/hexanes/Et₃N, 33/66/1, v/v/v).

^1H NMR (CDCl₃) δ 7.14 (t, J=7.96 Hz, 1H, H Aryl), 6.37-6.29 (m, 3H, H Aryl), 3.96 (bs, 1H, NH), 3.26 (s, 3H, NCH₃), 2.81 (s, 3H, NHCH₃). ^13C NMR (CDCl₃) δ 150.53 (ArNH), 141.46 (ArNCH₃), 130.17 (t-Ar), 114.50 (NCN), 107.72 (t-Ar), 103.12 (t-Ar), 98.58 (t-Ar), 36.70 (N(CH₃)CN), 30.43 (N(CH₃)H).

3.4.2.25. N-(3-((2-fluoroethyl)(methyl)amino)phenyl)-N-methylcyanamide (63)

To a stirred suspension of N-methyl-N-(3-(methylamino)phenyl)cyanamide (62) (397 mg, 2.46 mmol), potassium carbonate (342 mg, 2.48 mmol) and potassium iodide (41 mg, 0.25 mmol) in a mixture of dry THF and dry DMF (10 mL, 1/2, v/v) was added 1-bromo-2-fluoroethane (0.55 mL, 7.36 mmol). The reaction mixture was stirred at 80 °C for 24 h, before diluting with water (25 mL). The mixture was twice extracted with ethyl acetate (25 mL). The combined organic fraction was washed with water (25 mL) and brine (25 mL). The organic layer was collected, dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure. The crude product was purified over silica (dichloromethane/hexanes, 50/50, v/v) to obtain the title compound as yellow oil (157 mg, 0.76 mmol, 31%). R_f 0.55 (dichloromethane). ^1H NMR (CDCl₃) δ 7.21 (t, J=4.09 Hz, 1H, H Aryl), 7.15-6.35 (m, 3H, H Aryl), 4.57 (dt, J_HF=47.23, J=5.06 Hz, 2H, FCH₂), 3.62 (dt, J_HF=25.08, J=5.06 Hz, 2H, FCH₂), 3.26 (s, 3H, NCH₃CN), 2.99 (s, 3H, NCH₃CN). ^13C NMR (CDCl₃) δ 149.89 (ArN(CH₃)CH₂), 141.44 (ArN(CH₃)CN), 130.07 (t-Ar), 114.32 (NCN), 107.36 (t-Ar), 102.68 (t-Ar), 98.64 (t-Ar), 81.61 (d, J_CF=169.77 Hz), 52.38 (d, J_CF=20.82 Hz), 36.60 (N(CH₃)CN), 38.81 (N(CH₃)CH₂). ^19F NMR (CDCl₃) δ 18.18 (tt, J_HF=47.24, J_HF=24.92 Hz, FCH₂).

3.4.2.26. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-((2-fluoroethyl)(methyl)amino)phenyl)-1-methylguanidine (64)

The reaction of N-(3-((2-fluoroethyl)(methyl)amino)phenyl)-N-methylcyanamide (63) (257 mg, 1.00 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (176 mg, 0.84 mmol) in toluene (400 µL) according to procedure A at 140 °C afforded, after purification over silica with ethyl acetate/hexanes/Et₃N, 33/66/1, v/v/v, the title
compound as a light yellow glassy solid (154 mg, 0.40 mmol, 53%). 

\[ R_f \] 0.11 (ethyl acetate/hexanes/Et\(_3\)N, 33/66/1, v/v/v). 1H NMR (CDCl\(_3\)) \( \delta \) 7.29-7.22 (m, 2H, H\(_{Ar}\)), 6.94 (d, \( J = 2.274 \) Hz, 1H, H\(_{Ar}\)), 6.81 (dd, \( J = 8.34 \), 2.30Hz, 1H, H\(_{Ar}\)), 6.69-6.61 (m, 3H, H\(_{Ar}\)), 4.62 (dt, \( J_{CF}=47.21 \), 5.04Hz, 2H, FCH\(_2\)CH\(_2\)N), 3.59 (bs, 2H, NH), 3.66 (dt, \( J_{CF}=24.98 \), 5.06Hz, 2H, FCH\(_2\)CH\(_2\)N), 3.42 (s, 3H, NCH\(_3\)), 3.04 (s, 3H, FCH\(_2\)CH\(_2\)NCH\(_3\)), 2.46 (s, 3H, SCH\(_3\)). 13C NMR (CDCl\(_3\)) \( \delta \) 151.04 (FCH\(_2\)CH\(_2\)N(CH\(_3\))Ar), 150.06 (Ar-NCH\(_3\)), 147.67 (NCN), 145.37 (Ar-NH), 137.53 (Ar-S), 129.93 (Ar), 124.48 (Ar-Cl), 122.46 (Ar), 120.93 (Ar), 114.85 (Ar), 110.70 (Ar), 110.05 (Ar), 81.71 (d, \( J_{CF}=169.69 \)Hz, FCH\(_2\)CH\(_2\)N), 52.33 (d, \( J_{CF}=20.82 \)Hz, FCH\(_2\)CH\(_2\)N), 38.97 (FCH\(_2\)CH\(_2\)NCH\(_3\)), 38.67 (NCH\(_3\)), 15.85 (SCH\(_3\)). 19F NMR (CDCl\(_3\)) \( \delta \) 18.24 (tt, \( J = 94.14 \), 23.21Hz, 1F, FCH\(_2\)CH\(_2\)N). The product was converted into its fumaric acid salt, (161 mg, 0.36 mmol, 90 % yield) as white solid. 1H NMR (DMSO-\( d_6 \)) \( \delta \) 7.28 (d, \( J = 8.05 \)Hz, 1H, H\(_{Ar}\)), 7.18 (t, \( J = 8.00 \)Hz, 1H, H\(_{Ar}\)), 6.85-6.80 (m, 2H, H\(_{Ar}\)), 6.65-6.55 (m, 3H, H\(_{Ar}\)), 6.52 (s, 1H, NH), 5.64 (bs, 2H, NH), 4.58 (dt, \( J_{CF}=47.61 \), J=4.85Hz, 2H, FCH\(_2\)CH\(_2\)N), 3.65 (dt, \( J_{CF}=26.35 \), J=4.82Hz, 2H, FCH\(_2\)CH\(_2\)N), 3.26 (s, 3H, NCH\(_3\)), 2.94 (s, 3H, FCH\(_2\)CH\(_2\)NCH\(_3\)), 2.43 (s, 3H, SCH\(_3\)). HRMS [M + H]+ calcld for C\(_{18}\)H\(_{22}\)ClFN\(_4\)S 381.1310, found 381.1325. HPLC system A: purity 97.5%, rt = 5.03 min.

3.4.2.27.2,2,2-trifluoro-N-(2-fluoroethyl)-N-(3-(N-methylcyanamido)phenyl)acetamide (65)

To a suspension of potassium carbonate (279 mg, 2.02 mmol), potassium iodide (35 mg, 0.21 mmol) and 2,2,2-trifluoro-N-(3-(N-methylcyanamido)phenyl)acetamide (55) (492 mg, 2.02 mmol) in dry DMF (4 mL) and dry THF (2 mL) was added 1-bromo-2-fluoroethane (0.45 mL, 6.0 mmol). After stirring for 26 h at 80 °C, the reaction mixture was diluted with water (25 mL) and extracted twice with ethyl acetate (25 mL). The combined organic fraction was washed with brine (25 mL), collected and dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure. The crude product was purified over silica (gradient of ethyl acetate/hexanes 20/80 to 20/50, v/v) to obtain the title compound as a white solid (408 mg, 1.41 mmol, 70%). 

\[ R_f \] 0.40 (dichloromethane). 1H NMR (CDCl\(_3\)) \( \delta \) 7.46 (t, \( J = 8.40 \)Hz, 1H, H\(_{Ar}\)), 7.20-7.16 (m, 1H, H\(_{Ar}\)), 7.08-7.05 (m, 2H, H\(_{Ar}\)), 4.63 (dt, \( J_{HF}=47.17 \)Hz, J=4.75Hz, 2H, FCH\(_2\)CH\(_2\)Cl), 4.04 (dt, \( J_{HF}=25.87 \)Hz, J=4.71Hz, FCH\(_2\)CH\(_2\)Cl), 3.35 (s, 3H, NCH\(_3\)). 13C NMR (CDCl\(_3\)) \( \delta \) 156.66 (q, \( J_{CF}=36.13 \)Hz, CO), 141.50 (Ar-N), 140.25 (Ar-N), 130.45 (Ar), 122.96 (Ar), 115.96 (d, \( J_{CF}=288.26 \)Hz, CF\(_3\)), 115.48 (Ar), 114.62 (Ar), 113.04 (NCN), 79.85 (d, \( J_{CF}=170.23 \)Hz, FCH\(_2\)CH\(_2\)Cl), 51.91 (d, \( J_{CF}=20.16 \)Hz, FCH\(_2\)CH\(_2\)Cl), 36.65 (CH\(_3\)). 19F NMR (CDCl\(_3\)) \( \delta \) 16.44 (tt, \( J_{HF}=47.12 \)Hz, J\(_{HF}=25.83 \)Hz, CH\(_2\)CH\(_2\)F), -67.31 (s, CF\(_3\)).
3.4.2.28. N-(3-(3-(2-chloro-5-(methylthio)phenyl)-1-methylguanidino)phenyl)-2,2,2-trifluoro-N-(2-fluoroethyl)acetamide (66)

The reaction of 2,2,2-trifluoro-N-(2-fluoroethyl)-N-(3-(N-methylcyanamido)phenyl)acetamide (65) (204 mg, 0.71 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (164 mg, 0.78 mmol) in toluene (200 µL) according to procedure A at 140 °C afforded, after purification over silica with ethyl acetate/hexanes/Et3N (50/50/1, v/v/v), the title compound as a light yellow glassy solid (214 mg, 0.46 mmol, 66%). Rf 0.18 (ethyl acetate/hexanes/Et3N, 50/50/1, v/v/v). 1H NMR (CDCl3) δ 7.51-7.40 (m, 2H, H Aryl), 7.31-7.26 (m, 2H, H Aryl), 7.19-7.16 (m, 1H, H Aryl), 6.89 (d, J=2.24 Hz, 1H, H Aryl), 6.82 (dd, J=8.34 Hz, J=2.27 Hz, 1H, H Aryl), 4.65 (dt, JCF=47.17 Hz, J=4.68 Hz, 2H, FCH2CH2), 4.04 (dt, JCF=25.81 Hz, J=4.60 Hz, FCH2CH2), 4.03 (bs, 2H, NH), 3.42 (s, 3H, NCH3), 2.45 (s, 3H, SCH3). 13C NMR (CDCl3) δ 156.74 (q, JCF=54.16 Hz, CO), 150.49 (Ar-NCO), 146.98 (Ar-NH), 145.57 (NCH3), 139.87 (Ar-NH), 137.77 (Ar-S), 130.43 (Ar), 129.97 (Ar), 126.97 (Ar), 126.44 (Ar), 125.67 (Ar), 124.00 (Ar-Cl), 122.01 (Ar), 121.20 (Ar), 116.05 (d, JCF=288.34 Hz, CF3), 80.16 (d, JCF=170.55 Hz, FCH2CH2), 52.03 (d, JCF=20.11 Hz, FCH2CH2), 38.62 (NCH3), 15.69 (SCH3). 19F NMR (CDCl3) δ 16.93 (tt, JHF=47.15 Hz, JHF=25.90 Hz, CH2CH2F), -67.17 (s, CF3).

3.4.2.29. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(2-fluoroethylamino)phenyl)-1-methylguanidine (67)

To a solution of N-(3-(3-(2-chloro-5-(methylthio)phenyl)-1-methylguanidino)phenyl)-2,2,2-trifluoro-N-(2-fluoroethyl)acetamide (66) (214 mg, 0.46 mmol) in methanol/water (5 mL, 5/1, v/v) was added potassium carbonate (68 mg, 0.49 mmol) and stirred at room temperature for 1h. The reaction mixture was diluted with water (25 mL) and extracted twice with ethyl acetate (25 mL). The combined organic fraction was washed with brine (25 mL), dried over anhydrous magnesium sulfate, filtered and evaporated under reduced pressure. The crude product was purified over silica (gradient of dichloromethane to dichloromethane/methanol 95/5, v/v) to obtain the title compound as colorless oil (162 mg, 0.44 mmol, 96%). Rf 0.11 (ethyl acetate/hexanes/Et3N, 50/50/1, v/v/v). 1H NMR (CDCl3) δ 7.29-7.25 (m, 1H, H Aryl), 7.21 (t, J=7.92 Hz, 1H, H Aryl), 6.93 (d, J=2.29 Hz, 1H, H Aryl), 6.82 (dd, J=8.35 Hz, J=2.35 Hz, 1H, H Aryl), 6.70-6.66 (m, 1H, H Aryl), 6.59-6.52 (m, 2H, H Aryl), 4.64 (dt, JHF=47.30 Hz, J=4.85 Hz, 2H, FCH2CH2), 4.15 (t, J=5.67 Hz, 1H, FCH2CH2), 4.02 (bs, 2H, NH), 3.54-3.37 (m 2H, FCH2CH2), 3.40 (s, 3H, NCH3), 2.46 (s, 3H, SCH3). 13C NMR (CDCl3) δ 150.94 (Ar-NHCH2), 148.84 (Ar-NCH3), 147.57 (NCH3), 145.29 (Ar-NH), 137.52 (Ar-S), 130.37 (Ar), 129.90 (Ar), 124.42 (Ar-Cl), 122.36 (Ar), 120.86 (Ar), 115.91 (Ar), 111.22 (Ar), 82.26 (d, JCF=137.59 Hz, FCH2CH2), 43.86 (d, JCF=20.34 Hz, FCH2CH2), 38.52 (NCH3), 15.73 (SCH3). 19F NMR (CDCl3) δ 16.93 (tt, JHF=47.30, JHF=26.79 Hz, FCH2CH2NCH3). The free base was converted into its fumaric acid salt (150 mg, 0.32 mmol, 73%). 1H NMR
(DMSO-\textit{d}_6) \delta 7.31 (d, J=8.18Hz, 1H, H\textit{Aryl}), 7.09 (t, J=7.87Hz, 1H, H\textit{Aryl}), 6.91-6.86 (m, 2H, H\textit{Aryl}), 6.55 (s, 2H, fumaric acid), 6.54-6.47 (m, 3H, H\textit{Aryl}), 6.14 (bs, 1H, NH), 6.05 (bs, 2H, NH), 4.54 (dt, J_{HF}=47.62Hz, J=4.96Hz, 2H, F\textit{CH}_2\textit{CH}_2), 3.42-3.27 (m, 2H, F\textit{CH}_2\textit{CH}_2), 3.26 (s, 3H, N\textit{CH}_3), 2.44(s, 3H, S\textit{CH}_3). HRMS [M + H]^+ calcd for C\textsubscript{17}H\textsubscript{20}ClFN\textsubscript{4}S 367.1154, found 367.1159. HPLC system A: purity 99.7%, rt = 5.03 min.

3.4.2.30.2-bromoethyl-4-methylbenzenesulfonate (69)
A solution of \textit{p}-toluenesulfonyl chloride (955 mg, 5.01 mmol) in dichloromethane (2.5 mL) was added dropwise to a cooled (0 °C, ice-water bath) solution of 2-bromoethanol (68) in dichloromethane (2.5 mL) and pyridine (2.5 mL). After addition, the ice-water bath was removed and stirring was continued overnight. The reaction mixture was diluted with diethyl ether (25 mL) and washed with 1M HCl (50 mL), water (25 mL) and brine (25 mL). The organic fraction was collected and dried with anhydrous magnesium sulfate, filtered and evaporated under reduced pressure. The residue was purified over silica using ethyl acetate/hexanes (10/90, v/v) as eluent to obtain the title compound as a colorless oil (431 mg, 1.54 mmol, 31%).

R\textsubscript{f} 0.14 (ethyl acetate/hexanes, 10/90, v/v). \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \delta 7.77 (d, J=8.33Hz, 2H, H\textit{Aryl}), 7.33 (d, J=8.06Hz, 2H, H\textit{Aryl}), 4.25 (t, J=6.37Hz, 2H, CH\textsubscript{2}), 3.44 (t, J=6.37Hz, 2H, CH\textsubscript{2}), 2.42 (s, 3H, CH\textsubscript{3}); \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \delta 145.30 (Ar-S), 132.47 (Ar-CH\textsubscript{3}), 130.00 (Ar), 127.93 (Ar), 68.77 (CH\textsubscript{2}O), 27.52 (BrCH\textsubscript{2}), 21.65 (CH\textsubscript{3}).

3.4.2.31.N-\textit{N}-((2-hydroxyethyl)(methyl)amino)phenyl-N-methylcyanamide (72)
To a stirred suspension of \textit{N}-methyl-N-\textit{N}-(3-(methylamino)phenyl)cyanamide (62) (1.86 g, 11.5 mmol), \textit{N},\textit{N}-di-isopropylethylamine (2.00 mL, 11.5 mmol) and potassium iodide (0.19 g, 1.15 mmol) in a mixture of dry THF and dry DMF (50 mL, 1/2, v/v) was added 2-bromoethanol (0.81 mL, 11.5 mmol). The reaction mixture was stirred at 125 °C for 6 h, before diluting with water (150 mL). The mixture was extracted with ethyl acetate (2 x 75 mL). The combined organic fraction was washed with water (100 mL) and brine (50 mL). The organic layer was collected, dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure. The crude product was purified over silica (ethyl acetate/hexanes/\textit{Et}_3\text{N}, 33/66/1, v/v/v) to obtain the title compound as a light yellow oil (1.11 g, 4.92 mmol, 43%). R\textsubscript{f} 0.55 (ethyl acetate). \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \delta 7.15 (t, J=8.18Hz, 1H, H\textit{Aryl}), 6.47 (dd, J=8.33Hz, J=2.20Hz, 1H, H\textit{Aryl}), 6.41 (t, J=2.26Hz, 1H, H\textit{Aryl}), 6.33 (dd, J=7.95Hz, 2.10Hz, 1H, H\textit{Aryl}), 3.76 (t, J=5.86Hz, 2H, N\textit{CH}_2), 3.45 (t, J=5.88Hz, 2H, CH\textsubscript{2}OH), 3.26 (s, 3H, N\textit{CH}_3CN), 2.96 (s, 3H, CH\textsubscript{2}N\textsubscript{CH}_3), 2.65 (bs, 1H, OH). \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \delta 13C NMR (CDCl\textsubscript{3}) \delta 150.77 (Ar-N\textsubscript{CH}_3), 141.42 (Ar-N\textsubscript{CH}_3CN), 130.14 (Ar), 114.60 (NCN), 107.79 (Ar), 102.73 (Ar), 98.96 (Ar), 59.75 (N\textsubscript{CH}_2), 54.91 (CH\textsubscript{2}OH), 38.85 (CH\textsubscript{2}N\textsubscript{CH}_3), 36.78 (N\textsubscript{CH}_3CN).
The reaction of \( N-(3-((2-hydroxyethyl)(methyl)amino)phenyl)-N-methylcyanamide (72) \) (524 mg, 2.33 mmol) with 2-chloro-5-(methylthio)aniline hydrochloride (590 mg, 2.81 mmol) in toluene (500 µL) according to procedure A at 140 °C afforded, after purification over silica with dichloromethane/hexanes/Et₃N (gradient from 50/50/1 to 75/25/1, v/v/v), the title compound as a light yellow oil (504 mg, 1.33 mmol, 52%).

\[ \text{Rf} \ 0.35 \ \text{(ethyl acetate/Et}_3\text{N, 99/1, v/v).} \]

\[ ^1\text{H NMR (CDCl}_3\) \delta 7.20-7.08 (m, 2H, HAryl), 6.85 (d, \ J=2.24\text{Hz}, 1H, H_Aryl), 6.72 (dd, \ J=8.37\text{Hz}, J=2.27\text{Hz}, 2H, H_Aryl), 6.53-6.48 (m, 3H, H_Aryl), 3.99 (bs, 3H, NH & OH), 3.59 (t, \ J=5.94\text{Hz}, 2H, NCH₂), 3.30 (t, \ J=6.30\text{Hz}, 2H, CH₂OH), 3.29 (s, 3H, NCH₃CNH), 2.84 (s, 3H, CH₂NCH₃), 3.25 (SCH₃). \]

\[ ^{13}\text{C NMR (CDCl}_3\) \delta 151.55 (Ar-NCH₃), 150.42 (Ar-NCH₃), 147.11 (NCN), 144.77 (Ar-NH), 137.47 (Ar-S), 129.96 (Ar), 129.74 (Ar), 124.48 (Ar-Cl), 122.48 (Ar), 120.85 (Ar), 113.95 (Ar), 110.42 (Ar), 110.27 (Ar), 58.96 (NCH₂CH₂), 54.32 (NCH₂CH₂), 38.47 (2 x NCH₃), 15.47 (SCH₃). \]

3.4.2.33. ((3-(3-(2-chloro-5-(methylthio)phenyl)-1-methylguanidino)phenyl) (methyl)amino)ethy 4-methylbenzenesulfonate (74)

To a stirred solution of 3-(2-chloro-5-(methylthio)phenyl)-1-(3-((2-hydroxyethyl)(methyl)amino)phenyl)-1-methylguanidine (73) (504 mg, 1.33 mmol), triethylamine (570 µL, 4.09 mmol) and 4-dimethylaminopyridine (21 mg, 0.17 mmol) in dichloromethane (13.5 mL) was added \( p\)-toluenesulfonyl chloride (325 mg, 1.71 mmol). After 21 h at room temperature, the reaction mixture was washed with water (25 mL), aqueous NaHCO₃ (25 mL) and saturated NaHCO₃ (25 mL). The organic layer was collected, dried with anhydrous magnesium sulfate, filtrated and evaporated to dryness under reduced pressure. The crude product was purified over silica using dichloromethane/methanol (96/4, v/v) to obtain the title compound as a white solid (215 mg, 0.40 mmol, 24%).

\[ \text{Rf} \ 0.13 \ \text{(dichloromethane/methanol, 95/5, v/v).} \]

\[ ^1\text{H NMR (CDCl}_3\) \delta 7.72 (d, \ J=8.05\text{Hz}, 2H, H_Aryl-tosylate), 7.32-7.18 (m, 2H, H_Aryl-tosylate), 6.83-6.79 (m, 1H, H_Aryl), 6.66 (d, \ J=7.80\text{Hz}, 1H, H_Aryl), 6.56-6.49 (m, 2H, H_Aryl), 4.24 (bs, 2H, NH), 4.18 (t, \ J=5.35\text{Hz}, 2H, NCH₂CH₂O), 3.56 (t, \ J=5.35\text{Hz}, 2H, NCH₂CH₂O), 3.40 (s, 3H, NCH₃CNH), 2.91 (s, 3H, CH₂NCH₃), 2.45 (s, 3H, Ar-CH₃), 2.43 (s, 3H, SCl₂). \]

\[ ^{13}\text{C NMR (CDCl}_3\) \delta 151.11 (Ar-NCH₃), 149.38 (Ar-NCH₃), 147.15 (NCN), 145.10 (Ar-SO₂), 144.89 (Ar-NH), 137.51 (Ar-S), 132.39 (Ar-CH₃), 130.22 (Ar), 129.82 (Ar), 129.76 (Ar), 127.67 (Ar), 124 (Ar-Cl), 122.41 (Ar), 120.95 (Ar), 114.78 (Ar), 110.45 (Ar), 110.14 (Ar), 66.84 (NCH₂CH₂O), 50.87 (NCH₂CH₂O), 38.71 (NCH₃), 38.63 (NCH₃), 21.52 (Ar-CH₃), 15.71 (SCH₃). \]

HRMS [M + H]⁺ calcld for C₂₅H₂₉ClN₄O₃S₂ 533.1442, found 533.1523.
### 3.4.3. Radiochemistry

#### 3.4.3.1. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(N-[11C]methyl-N-methyl-amino)phenyl)-1-methylguanidine ([11C]59)

[11C]CO₂ was trapped into a solution of LiAlH₄ in THF (0.1 mL) at room temperature by a helium flow of 10 mL·min⁻¹. The solution was heated to 130 °C and the helium flow was increased to 100 mL·min⁻¹ to evaporate the THF. After 3 min the helium flow was adjusted to 10 mL·min⁻¹, HI (55% solution, 0.2 mL) was added and [11C]CH₃I was passed through a preheated silver triflate column of 200 °C, to obtain online [11C]CH₃OTf. [11C]CH₃OTf was trapped into the second reaction vial containing precursor 61 (0.5 mg, 1.40 µmol) and acetonitrile (300 µL). After complete trapping of [11C]CH₃OTf the reaction mixture was heated at 40 °C for 1 min. The reaction mixture was quenched with 10 mM ammonium hydrogen carbonate (pH=7.4, 1 mL) solution before loading it onto preparative HPLC (Reprosphere 100-C18-DE, 5μm, 80 x 8 mm, 10 mM ammonium hydrogen carbonate pH=7.4 / acetonitrile 30/70 v/v, 4 mL·min⁻¹, 254 nm). The product eluted at 7 min, was collected and diluted with water (40 mL). The solution was concentrated on a tC18plus Seppak, rinsed with water (20 mL), subsequently eluted with ethanol (96%, 1.5 mL) and diluted with a solution of 7.11 mM NaH₂PO₄ in 0.9% NaCl (w/v in water), pH=5.2 (13.5 mL) to give a final solution of 9.6% ethanol in phosphate buffer containing [11C]59. The (radio)chemical purity was verified using analytical radio HPLC (Alltima C18, 5μm, 250 x 4.6 mm, 20 mM potassium hydrogen phosphate pH=6.7 / methanol / acetonitrile 20/20/60 v/v/v, 1.0 mL·min⁻¹, 254 nm). The product eluted at 8.12 min. The specific activity was calculated against a calibration curve of 59 using the same HPLC system.

#### 3.4.3.2. 3-(2-chloro-5-(methylthio)phenyl)-1-methyl-1-(3-(11C)methylamino)phenyl)guanidine ([11C]61)


#### 3.4.3.3. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-((2-[18F]fluoroethyl)(methyl)amino)phenyl)-1-methylguanidine ([18F]64)

After irradiation, [18F]fluoride was trapped on a PS-HCO₃⁻ column. It was eluted from the anion exchange column into a screw cap reaction vessel with 1 mL of acetonitrile/water (9/1, v/v) containing 13 mg (34.5 µmol) of Kryptofix 2.2.2 and 3 mg (21.7 µmol) of potassium carbonate. The solution was evaporated to dryness under a helium flow (50 mL·min⁻¹) and reduced pressure at 90 °C. Acetonitrile (0.5 mL) was added and evaporated again. After cooling to room temperature, precursor
(74, 2.5 mg, 4.7 µmol) dissolved in acetonitrile (0.5 mL) was added and heated to 80 °C for 30 minutes. The reaction vessel was cooled to 30 °C and the reaction mixture was quenched with 25 mM ammonium dihydrogen phosphate (1 mL, pH 2.5) solution. The reaction mixture was purified by semi-preparative HPLC (Reprosphere 100-C18-DE, 5µm, 80 x 8 mm, 10 mM ammonium hydrogen carbonate pH=7.4 / acetonitrile 30/70 v/v, 4 mL·min⁻¹, 254 nm). The product eluted at 19 min, was collected and diluted with water (30 mL). The solution was concentrated on a tC18plus Seppak, rinsed with water (20 mL), subsequently eluted with ethanol (96%, 1.5 mL) and diluted with a solution of 7.11 mM NaH₂PO₄ in 0.9% NaCl (w/v in water), pH=5.2 (13.5 mL) to give a final solution of 9.6% ethanol in phosphate buffer containing [¹⁸F]64. The (radio)chemical purity was verified using analytical radio HPLC (Alltima C18, 5µm, 250 x 4.6 mm, 20 mM potassium hydrogen phosphate pH=6.7 / methanol / acetonitrile 20/20/60, v/v/v, 1.0 mL·min⁻¹, 254 nm). The product eluted at 7.48 min. The specific activity was calculated against a calibration curve of 64 using the same HPLC system.

3.4.4. Determination of LogDₗoct,7.4

The distribution of the radiolabeled compounds between 1-octanol and 0.2M phosphate buffer (pH = 7.4) was measured in triplicate at room temperature. Briefly, 1 mL of a 1-5 MBq·mL⁻¹ solution of the radiolabeled compound in 0.2M phosphate buffer (pH 7.4) was vigorously mixed with 1 mL of 1-octanol for 1 min at room temperature using a vortex. After a settling period of 30 minutes, five samples of 100 µL were taken from both layers. For determining recovery, 5 samples of 100 µL were taken from the 1-5 MBq·mL⁻¹ solution. All samples were counted for radioactivity. The LogDₗoct,7.4 value was calculated according to LogDₗoct,7.4 = 10Log(Aₗoct / Aₗbuffer), where Aₗoct and Aₗbuffer represent average radioactivity of 5 1-octanol and 5 buffer samples, respectively.
3.4.5. Pharmacology

3.4.5.1. Animals

All animal experiments were performed in compliance with Dutch laws on animal experimentation and after approval by the local animal ethics committee, study number DEC_PET12-01.

Adult male Wistar rats (Harlan, Horst, The Netherlands) weighing 200 to 224 g at arrival were group housed with five to six animals per cage and had *ad libitum* access to food (Teklad Global 16% Protein Rodent Diet, Harlan, Madison, WI, USA) and tap water. Rats were kept in an animal room with a constant temperature of ~21°C and in a 12-h light/dark cycle in which lights were switched on at 8:00 am.

3.4.5.2. Membrane preparation

Male Wistar rats (150-200 g) were killed by decapitation. The forebrains were rapidly removed and homogenized using a DUALL tissue homogenizer (10 strokes, 2,000 rpm), in a 7-fold excess (v/w) of ice-cold 0.25 M sucrose. The nuclei and cell debris were removed by centrifugation (10 min x 400 x g) in a Sorvall RC-6 refrigerated centrifuge (rotor SA600). The supernatant was decanted and the resulting pellet was rehomogenized in 5 vol. 0.25 M sucrose and recentrifuged. The combined supernatants were diluted in Tris-acetate buffer (50 mM, pH 7.4) to a final dilution of 40 v/w, and centrifuged for 30 min x 30,000 x g, in order to obtain membranes from the cell surface, mitochondrial, and microsomal fractions. The pellet was resuspended in 20 vol. of 50 mM Tris buffer containing 0.04 % Triton X-100 (pH 7.4), and was kept at 25 °C for 2 hr before recentrifugation. The resulting pellet was suspended in Tris-HCl buffer (dilution 4, pH 7.4) and stored at -80 °C in 5 ml aliquots. On the day of each experiment, membranes were thawed to room temperature and washed twice with Tris buffer by centrifugation (30 min x 48,000 x g). After the final centrifugation step, pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) and further diluted to 40 vol. of buffer per g original weight wet tissue for competition binding experiments. Protein concentration was determined using the BCA protein kit (Sigma-Aldrich, The Netherlands).

3.4.5.3. Competition binding assays

*In vitro* competition binding experiments were performed using 5 nM [³H]MK-801 (specific activity 22.5 Ci/mmol; PerkinElmer, USA). All compounds were dissolved as 10 mM stock solutions in DMSO, and used in a concentration range from 10⁻⁴ to 10⁻¹² M, with a DMSO concentration of maximal 1%. Competition binding experiments were conducted at room temperature, in a final volume of 500 µL assay buffer (50 mM Tris-HCl, pH 7.4), containing 1 µM L-glutamate and glycine. The incubation mixture was
composed of 400 µL membrane suspension (protein concentration 1 µg·µL⁻¹), 50 µL [³H]MK-801, 45 µL assay buffer and 5 µL unlabeled drug solution. Nonspecific binding was determined in the presence of 30 µM 5. Incubations were terminated after 17 hr by filtration, using a 48-well Brandel harvester and Whatman GF/B filters, presoaked in 0.3% polyethyleneimine. The filters were washed three times with 3 mL of ice-cold Tris-HCl buffer (pH 7.4), and subsequently radioactivity was determined by liquid scintillation spectrometry in 5 mL of Optiphase-HiSafe 3, at an efficiency of 40%.

3.4.5.4. Data analysis

Kᵢ values were determined by nonlinear regression analysis using the equation: logEC50 = log[10(logKi*(1+RadioligandNM/HotKdNM))], (GraphPad Software Inc, San Diego, CA).

3.4.5.5. Biodistribution and blocking studies

The biodistribution of [¹¹C]⁵⁹, [¹¹C]⁶¹ and [¹⁸F]⁶⁴ was determined in male Wistar rats. Rats (N=4 per time point) received a dose of either 100 MBq [¹¹C]⁵⁹ and [¹¹C]⁶¹ or either 40 MBq [¹⁸F]⁶⁴ in the tail vein without anesthesia and were decapitated at either 5, 15, 30 or 60 min after injection. Blood, heart, lung, liver, kidney and bone were removed. In addition, several brain areas were dissected: hippocampus, striatum, hypothalamic region, cerebellum, cerebral cortex and rest brain. These tissues were then counted for radioactivity with a gamma counter (LKB Wallac, Turku, Finland) and weighed. Results were expressed as percent injected dose per gram (% ID/g). For blocking studies, rats received 15 minutes prior injection of radiotracer a dose of 0.6 mg·kg⁻¹ MK-801 intraperitoneal.

3.4.5.6. Metabolite analysis

To study the metabolic profile of [¹¹C]⁵⁹, [¹¹C]⁶¹ and [¹⁸F]⁶⁴ in the plasma and brain, six rats were injected with either 100 MBq [¹¹C]⁵⁹ and [¹¹C]⁶¹ or either 50 MBq [¹⁸F]⁶⁴ in the tail vein without anesthesia. After 15 or 45 minutes the rats were decapitated. A blood sample was obtained and the brain was isolated. All blood samples were collected in heparin tubes and centrifuged at 4000 rpm for 5 min at 4°C (Hettich Universal 16, Depex B.V, The Netherlands). Plasma was separated from blood cells, diluted with 2 mL of water containing 20 µL of 37% hydrochloric acid, was loaded onto a tC² Plus Sep-Pak (Waters, The Netherlands) and washed with 5 mL of water. The eluate was defined as the polar metabolite fraction. Subsequently, the tC² Plus Sep-Pak was eluted with 1.5 ml of methanol containing 0.1% diisopropylamine. This eluate was defined as the non-polar fraction and was analyzed using HPLC (Phenomenex Gemini C8, 250x10.0 mm, 0.1M ammonium acetate / acetonitrile, gradient, 0-3 minutes 10% Acetonitrile, 11 minutes 80% Acetonitrile, 14 minutes 10%
acetonitrile, 4.0 mL·min⁻¹). Brain tissue was homogenized with an ultrasonic homogenizer (Braunsonic 1510, Kronberg, Germany) in water, under ice cooling, and subsequently centrifuged at 4000 rpm for 5 min at 4°C. Separated supernatants were loaded onto a tC2 Plus Sep-Pak and treated as above. The recovery of the metabolite analysis was validated to be above 95%.

3.5. Acknowledgements

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3.6. Supplementary data

Figure S1. HPLC chromatograms of [¹¹C]59. The right hand side shows the semi-preparative purification, the left hand side quality control. Both upper panels represent the UV-channel and the lower panels the radioactivity channel.
Figure S2. HPLC chromatograms of $^{[1]}$C61. The right hand side shows the semi-preparative purification, the left hand side quality control. Both upper panels represent the UV-channel and the lower panels the radioactivity channel.

Figure S3. HPLC chromatograms of $^{[18]}$F64. The right hand side shows the semi-preparative purification, the left hand side quality control. Both upper panels represent the UV-channel and the lower panels radioactivity channel.
3.7. References


Synthesis, radiolabeling and preclinical evaluation of a $^{11}$C-GMOM derivative as PET radiotracer for the ion channel of the N-methyl-D-aspartate receptor

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Abstract

Introduction: Presently available PET ligands for the NMDAr ion channel generally suffer from fast metabolism. The purpose of this study was to develop a metabolically more stable ligand for the NMDAr ion channel, taking $[^{11}C]_{\text{GMOM}}$ ($[^{11}C]5$) as the lead compound.

Methods: $[^{11}C]5$, its fluoralkyl analogue $[^{18}F]_{\text{PK209}}$ ($[^{18}F]37$) and the newly synthesized fluorovinyloxy analogue $[^{11}C]78b$ were evaluated ex vivo in male Wistar rats for metabolic stability. In addition, $[^{11}C]78b$ was subjected to a biodistribution study and its affinity ($K_i$) and lipophilicity ($\log D_{7.4}$) values were determined.

Results: The addition of a vinyl chain in the fluoromethoxy moiety did not negatively alter the affinity of $[^{11}C]78b$ for the NMDAr, while lipophilicity was increased. Biodistribution studies showed higher uptake of $[^{11}C]78b$ in forebrain regions compared with cerebellum. Pre-treatment with MK-801 decreased the overall brain uptake significantly, but not in a region-specific manner. 45 minutes after injection 78, 90 and 87% of activity in the brain was due to parent compound for $[^{11}C]5$, $[^{18}F]37$ and $[^{11}C]78b$, respectively. In plasma, 26 – 31% of activity was due to parent compound.

Conclusion: Complete substitution of the alpha-carbon increased lipophilicity to more favourable values. Substitution of one or more hydrogens of the alpha-carbon atom in the methoxy moiety improved metabolic stability. In plasma, more parent compound was found for $[^{18}F]37$ and $[^{11}C]78b$ then for $[^{11}C]5$, although differences were not significant. At 45 minutes, significantly more parent $[^{18}F]37$ and $[^{11}C]78b$ was measured in the brain compared with $[^{11}C]5$. 
4.1. Introduction

The N-methyl-D-aspartate receptor (NMDAr) is besides the AMPA and kainate receptors one of the three members of the ionotropic glutamate receptor family, named after their corresponding agonists. The NMDArs are formed as tetraheteromers, and contain an obligatory glycine-binding NR1 subunit, in complex with glutamate-binding NR2A-D, and occasionally NR3A-B subunits. These subunits together form a ligand-gated ion channel, within which a magnesium binding site exists. At resting state, the ion channel is blocked by Mg$^{2+}$, which inhibits ion flow (Na+, K+ and Ca$^{2+}$) through the channel. This Mg$^{2+}$ block can be removed by depolarization of the plasma membrane, a physiological process underlying the important role of NMDArs in synaptic plasticity, learning and memory. Deregulation of NMDArs is involved in several neurological and neuropsychiatric disorders [1,2,3].

Phenylcyclidine (PCP), thienylcyclohexyl piperidine (TCP), ketamine, memantine and MK-801 are compounds known to bind within the ion-channel site of NMDArs and block the channel pore. These compounds were initially synthesized for therapeutic purposes, and later radiolabeled to develop PET or SPECT radiotracers for the NMDArs [4,5,6,7].

Another class of interesting compounds that interacts at the ion channel binding site are the $N,N'$-diarylguanidines. To date, several $N$-(2,5-disubstituted phenyl)-$N'$-(3-substituted phenyl)-$N'$-methylguanidines, such as $[^{11}C]$GMOM, $[^{11}C]$CNS-5161, $[^{18}F]$GE-179 and $[^{18}F]$PK209 ($[^{18}F]$37), have been reported, all with promising characteristics, such as high affinity, moderate lipophilicity, and selectivity for the NMDA ion channel over the sigma receptor.

$[^{11}C]$GMOM, ([$^{11}C$]5, $K_i$: 5.2 – 21.7 nM vs. $[^3H]$MK-801; log P: 2.34; logD$_{7.4}$: 1.72) [8,18] demonstrated regional brain uptake in awake rats ranging from 0.75 ± 0.13 % injected dose per gram (ID/g) in the medulla and pons to 1.15 ± 0.17 % ID/g in the occipital cortex. Pre-treatment with MK-801 (1 mg·kg$^{-1}$) significantly reduced $[^{11}C]$5 uptake in all regions (24-28%), while D-serine, a glycine site co-agonist, increased uptake in all regions (10-24%). Administering the NR2B subunit antagonist RO 25-6981 reduced uptake of $[^{11}C]$5 by 24-38% over control. In isoflurane anesthetized baboons a fairly uniform distribution volume across the brain was observed. Pre-treatment with MK-801 (0.5 and 1.0 mg·kg$^{-1}$) did not alter regional distribution volumes, indicating a lack of saturable binding. During scans the amount of $[^{11}C]$5 in plasma was, on average, 18.6 ± 5.9%, and only polar metabolites were observed [8,9].

In human, highest uptake of $[^{11}C]$GMOM was observed in NMDAr rich regions, such as hippocampus and thalamus. Lowest values were found in temporal cortex and cerebellum. $[^{11}C]$GMOM showed rapid metabolism, after 20 min. 50% of the
radioactivity in the plasma was due to parent compound. After administering S-ketamine, a significant reduction in uptake was observed for the entire brain suggesting specific binding of $[^{11}\text{C}]{\text{GMOM}}$ to NMDAr. $[^{11}\text{C}]{\text{GMOM}}$ could be used for imaging and quantifying the NMDAr [10].

$[^{11}\text{C}]{\text{CNS-5161}}$, ($[^{11}\text{C}]\text{6}$, $K_i$: 1.87 nM vs. $[^{3}\text{H}]{\text{MK-801}}$) a thiomethyl analogue of GMOM, with a logD$_{7,4}$ of 2.68, demonstrated uptake in human brain tissues with the lowest uptake in cerebellum and the highest in putamen and thalamus. This compound, however, suffered from fast metabolism and specific uptake could not be demonstrated [11,12,13,14,15].

In humans, $[^{18}\text{F}]\text{GE-179}$ ($[^{18}\text{F}]\text{7}$, $K_i$: 2.35 nM vs. $[^{3}\text{H}]{\text{TCP}}$), a fluoroethyl analogue of CNS-5161 with a logD$_{7,4}$ of 2.49, showed rapid plasma metabolism. After 16 min. 50% of the radioactivity in the plasma could be attributed to radiolabeled metabolites. Nevertheless, the rate of metabolism was slower than for $[^{11}\text{C}]{\text{CNS-5161}}$. Uptake in grey matter was relatively homogeneous [16,17].

Recently, $[^{18}\text{F}]\text{PK209}$ ($[^{18}\text{F}]\text{37}$, $K_i$: 18 nM vs, $[^{3}\text{H}]{\text{MK-801}}$), a fluoromethoxy analogue of GMOM with a logD$_{7,4}$ of 1.45 was reported. Preclinical evaluation in rhesus monkeys showed retention in NMDAr rich cortical regions relative to cerebellum. In two out of three subjects, a reduction in signal relative to baseline was seen after MK-801 pretreatment. However, $[^{18}\text{F}]\text{37}$ suffered from fast metabolism, as 10 min. post injection only 30 ± 6% of the radioactivity in the plasma was due to parent compound [18,19].

All compounds described above consist of the same guanidine core structure, and show fast peripheral metabolism. Structural alterations exist in one or more short alkyl chains. It is known that the P450 cytochrome system is responsible for O-, N- and S-demethylation and/or oxidation [20]. A common procedure to prevent metabolism of short alkyl chains is the replacement of hydrogen by deuterium or fluoride [21]. The CH$_2$F group can be used as a bio-isostere of a methyl group, preventing metabolic oxidation by its electron withdrawing effect [22].

Since $[^{18}\text{F}]\text{37}$ next to $[^{11}\text{C}]\text{5}$ still showed relatively fast metabolism in rhesus monkeys, an obvious choice was to introduce deuterium or more fluorine atoms in the methoxy moiety. From previous SAR studies it was known that there is some spatial freedom for altering the methoxy moiety without losing affinity towards the NMDAr. The methoxy moiety can be altered with two or three fluorine atoms, but the resulting molecules showed less promising characteristics for use as a PET tracer [18]. A vinylic moiety could be an alternative way to prevent metabolic oxidation of the methoxy moiety of $[^{18}\text{F}]\text{37}$. 
The aim of the present study was to develop an analogue of $[^{11}C]$GMOM with improved metabolic stability and to evaluate its potential as an NMDAr ion channel ligand.

![Chemical structures of $[^{11}C]$GMOM and $[^{18}F]$PK209](image)

Figure 1. $[^{11}C]$GMOM and its fluorinated analogue $[^{18}F]$PK209.

4.2. Materials and methods

4.2.1. General

Nuclear magnetic resonance (NMR) spectra ($^1H$, $^{13}C$, $^{19}F$ NMR) were recorded on a Bruker Avance 250 (250.13, 62.90, 235.36 MHz respectively) (Billerica, USA). Chemical shifts of the NMR spectra are reported in parts per million (ppm) relative to the solvent residual peak. Description of signals: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, ddd = doublet of doublets, dd, dt = doublet of triplets, tt = triplet of triplets, dq = double quartet. Thin-layer chromatography (TLC) was performed on Merck DC-alufolien, silica gel 60, F254. Flash column chromatography was performed on silica gel 60Å, 230 – 400 Mesh. All chemicals were used without further purification, unless stated otherwise. The high-performance liquid chromatography (HPLC) analysis system consisted of a Jasco PU-2089 HPLC pump (Jasco Benelux, de Meern, the Netherlands), a Rheodyne injector with a 20 μL loop (Thermo Fischer Scientific, Breda, the Netherlands), a Jasco UV-2075 Plus UV detector set at a wavelength of 254 nm and a Raytest Na(I) radioactivity detector (Raytest, Straubenhardt, Germany). HPLC data were collected and integrated with the software package GINA 5.01. For high-resolution mass spectrometry (HRMS), a Bruker MicroTOFQ with ESI (electrospray ionisation) in a positive mode (Billerica, USA) was used. Samples were injected (10 μL) in a liquid flow of methanol/water (1/1) at a rate of 100 μL·min$^{-1}$. $[^{11}C]$CO$_2$ was produced by the $^{14}$N(p,α)$^{11}$C nuclear reaction with an IBA Cyclone 18/9 cyclotron (Louvain-La-Neuve, Belgium). Radioactivity was measured with a Veenstra VDC-405 dose calibrator (Comecer, Joure, the Netherlands). Radiochemistry was carried out in homemade, remotely controlled devices [23].
4.2.2. Chemistry

4.2.2.1. N-(3-hydroxyphenyl)cyanamide (76a) and N-(3-hydroxyphenyl)-N-methyl cyanamide (76b)

N-(3-hydroxyphenyl)cyanamide (76a) and N-(3-hydroxyphenyl)-N-methyl cyanamide (76b) were prepared according to published procedures [18].

4.2.2.2. 1-(2-chloro-5-(methylthio)phenyl)-3-(3-hydroxyphenyl)guanidine (77a)

A screw cap reaction vessel was charged with N-(3-hydroxyphenyl)cyanamide (76a) (0.68 g, 5.08 mmol), 2-chloro-5-(methylthio)aniline hydrochloride (1.16 g, 5.51 mmol) and 200 µL chlorobenzene. Next, this vessel was flushed with nitrogen, closed and stirred at 165 °C for 12 h. The reaction mixture was cooled down to 20 °C and dissolved in ethyl acetate (25 mL) and washed with 0.1M HCl (2 x 25 mL) followed by water (25 mL). The pH of the combined aqueous layers was adjusted with potassium carbonate to pH ≥ 10 and extracted with ethyl acetate (2 x 25 mL). The organic layers were collected and dried over anhydrous MgSO₄, filtrated and evaporated to dryness under reduced pressure. The residue was purified by column chromatography using ethyl acetate / triethylamine (99:1, v/v) to obtain the title compound as a white solid (1.16 g, 3.77 mmol, 74%). Rf 0.13 (ethyl acetate / triethylamine 99:1 v/v). ¹H NMR (CDCl₃) δ 7.23 (d, J=8.40Hz, 1H, H Aryl), 7.05 (t, J=8.03Hz, 1H, H Aryl), 6.96 (d, J=2.21Hz, 1H, H Aryl), 6.84 (dd, J=8.43Hz, J=2.25Hz, 1H, H Aryl), 6.74–6.72 (m, 1H, H Aryl), 6.61–6.57 (m, 2H, H Aryl), 5.96 (bs, 4H, 3 x NH, OH), 2.38 (s, 3H, SCH₃); ¹³C NMR (CDCl₃) δ 157.76 (Ar-NH), 150.74 (NCN), 143.98 (Ar-O), 140.83 (Ar-NH), 137.93 (Ar-S), 130.02 (Ar), 129.99 (Ar), 124.60 (Ar-Cl), 122.55 (Ar), 121.87 (Ar), 113.67 (Ar), 111.43 (Ar), 109.69 (Ar), 15.55 (CH₃).

4.2.2.3. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyphenyl)-1-methylguanidine (77b)

3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyphenyl)-1-methylguanidine (77b) was prepared according to a published procedure [18].

4.2.2.4. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-fluorovinyloxy)phenyl)-guanidine (78a)

A screw cap reaction vessel containing a solution of sodium hydride (60%) (53 mg, 1.33 mmol) in 1,2-dimethoxyethane (2 mL) and N,N-dimethylformamide (0.15 mL) was stirred at 0 °C. 1-(2-chloro-5-(methylthio)phenyl)-3-(3-hydroxyphenyl)guanidine (77a) (194 mg, 0.63 mmol) and sodium iodide (19 mg, 0.13 mmol) was added. After 30 min. 1-bromo-1-fluoroethene (150 µL, 2.00 mmol) dissolved in 1,2-dimethoxyethane (0.5 mL) was added. Stirring at 60 °C was continued for 24 h. The reaction mixture was diluted with water (25 mL) and extracted with dichloromethane
(3 x 25 mL). The combined organic fraction was washed with water (25 mL) and brine (25 mL). The organic layer was collected, dried over anhydrous magnesium sulphate, filtered and evaporated to dryness under reduced pressure. The crude product was purified over silica (ethyl acetate / hexanes / triethylamine (33/66/1, v/v/v)) to obtain the title compound as light yellow oil. (41 mg, 0.12 mmol, 18%). RF 0.24 (ethyl acetate / hexanes / triethylamine 33/66/1 v/v/v). 1H NMR (CDCl3) δ 7.29-7.16 (m, 3H, H Aryl), 7.02-6.98 (m, 2H, H Aryl), 6.88-6.79 (m, 2H, H Aryl), 5.22 (bs, 3H, NH), 4.06 (t, JHF=3.62Hz, 1H, FCCHa), 3.81 (dd, JHF=38.58Hz, JHH=3.79Hz, FCCHb), 2.44 (s, 3H, SCH3); 13C NMR (CDCl3) δ 160.13 (d, JCF=274.83Hz, CF), 154.86 (Ar-NH), 149.13 (NCN), 148.94 (Ar-O), 143.15 (Ar-NH), 138.28 (Ar-S), 130.46 (Ar), 130.13 (Ar), 123.87 (Ar-Cl), 121.97 (Ar), 121.87 (Ar), 118.31 (Ar), 117.74 (Ar), 70.55 (d, JCF=30.79Hz, FC=CH2), 15.90 (SCH3); 19F NMR (CDCl3) δ -81.28 (dd, JHF=38.64, 3.22Hz, 1F, CF).

4.2.2.5. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(1-fluorovinyloxy)phenyl)-1-methyl guanidine (78b)

A screw cap reaction vessel containing a solution of sodium hydride (60%) (41 mg, 1.03 mmol) in 1,2-dimethoxyethane (2 mL) and N,N-dimethylformamide (0.15 mL) was stirred at 0 °C. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-hydroxyphenyl)-1-methylguanidine (77b) (154 mg, 0.48 mmol) and sodium iodide (16 mg, 0.11 mmol) was added. After 30 min. 1-bromo-1-fluoroethene (150 µL, 2.00 mmol) dissolved in 1,2-dimethoxyethane (0.5 mL) was added. Stirring at 60 °C was continued for 24 h. The reaction mixture was diluted with water (25 mL) and extracted with dichloromethane (3 x 20 mL). The combined organic fraction was washed with water (25 mL) and brine (25 mL). The organic layer was collected, dried over anhydrous magnesium sulphate, filtered and evaporated to dryness under reduced pressure. The crude product was purified over silica (ethyl acetate / hexanes / triethylamine (25/75/1, v/v/v)) to obtain the title compound as a yellow oil. (131 mg, 0.36 mmol, 75%). RF 0.21 (dichloromethane / methanol 95:5 v/v). 1H NMR (CDCl3) δ 7.37 (t, J=8.05Hz, 1H, H Aryl), 7.26 (d, J=8.24Hz, 1H, H Aryl), 7.15-7.09 (m, 2H, H Aryl), 7.02-6.97 (m, 1H, H Aryl), 6.89 (d, J=2.28Hz, 1H, H Aryl), 6.81 (dd, J=8.34, J=2.32Hz, 1H, H Aryl), 4.16-4.13 (m, 1H, FCHa), 3.95 (bs, 2H, NH), 3.90 (dd, JHF=38.20, 3.83Hz, 1H, FCHb), 3.41 (s, 3H, NCH3), 2.45 (s, 3H, SCH3); 13C NMR (CDCl3) δ 161.79 (Ar-NH), 157.39 (NCN) 152.79 (d, JCF=269.93Hz, CF), 147.29 (Ar-O), 146.10 (Ar-NH), 137.85 (Ar-S), 130.74 (Ar), 130.17 (Ar), 124.38 (Ar-Cl), 122.71 (Ar), 122.37 (Ar), 121.87 (Ar), 116.13 (Ar), 115.43 (Ar), 71.54 (d, JCF=30.66Hz, FC=CH2), 38.91 (NCH3), 16.01 (SCH3); 19F NMR (CDCl3) δ -81.41 (dd, JHF=38.18, 3.08Hz, 1F, CF). The free base (56 mg) was converted into its fumaric acid salt (39 mg, 0.08 mmol, 52%). 1H NMR (DMSO-d6) δ 1H NMR (DMSO-d6) δ 7.39 (t, J=8.34Hz, 1H, H Aryl), 7.28 (d, J=8.20Hz, 1H, H Aryl), 7.18-7.16 (m, 2H, H Aryl), 6.94 (d, J=8.20Hz, 1H, H Aryl), 6.85-6.78 (m, 2H, H Aryl), 6.59 (s, 1.7H, fumaric acid), 5.98 (bs, 2H,
4.2.3. Radiochemistry

4.2.3.1. $[^{11}\text{C}]5$ and $[^{18}\text{F}]37$

$[^{11}\text{C}]5$ and $[^{18}\text{F}]37$ were prepared according to published procedures [10,18].

4.2.3.2. 3-(2-chloro-5-(methylthio)phenyl)-1-(3-(1-fluorovinyloxy)phenyl)-1-$[^{11}\text{C}]$methylguanidine ($[^{11}\text{C}]78b$)

$[^{11}\text{C}]\text{CO}_2$ was trapped into a solution of LiAlH$_4$ in THF (0.1 mL) at room temperature by a helium flow of 10 mL·min$^{-1}$. The solution was heated to 130 °C and the helium flow was increased to 100 mL·min$^{-1}$ to evaporate the THF. After 3 min, the helium flow was adjusted to 10 mL·min$^{-1}$, HI (55% solution, 0.2 mL) was added and $[^{11}\text{C}]\text{CH}_3$I was trapped into a second reaction vial containing precursor $78a$ (0.5 mg, 1.40 µmol), 5M NaOH (5 µL) and DMSO (300 µL). After complete trapping of $[^{11}\text{C}]\text{CH}_3$I the reaction mixture was heated at 60 °C for 3 min. The reaction mixture was quenched with a 10 mM ammoniumhydrogencarbonate (pH=7.4, 0.8 mL) solution before loading it onto preparative HPLC (Platinum EPS, C18 100A, 5µ; 150x10.0 mm; 10 mM ammoniumhydrogencarbonate pH=7.4 / acetonitrile 25/75 v/v; 5 mL·min$^{-1}$; 254 nm). The product eluted at 7 min, and was collected and diluted with water (40 mL). The solution was concentrated on a tC18plus Seppak, rinsed with water (20 mL), subsequently eluted with ethanol (96%, 1.5 mL) and diluted with a solution of 7.11 mM NaH$_2$PO$_4$ in 0.9% NaCl (w/v in water), pH 5.2 (13.5 mL) to give a final solution of 9.6% ethanol in phosphate buffer containing $[^{11}\text{C}]78b$. The (radio)chemical purity was verified using analytical radio HPLC (Waters µBondapak, C18 125A, 10µm, 250x4.6 mm; 10 mM ammoniumhydrogencarbonate pH=7.4 / acetonitrile 30/70 v/v; 1.5 mL·min$^{-1}$, 254 nm). The specific activity was calculated against a calibration curve of $78b$ using the same HPLC system.

4.2.3.3. Determination of Log$D_{oct,7.4}$

The distribution of the radiolabeled compounds between 1-octanol and 0.2M phosphate buffer (pH = 7.4) was measured in triplicate at room temperature. Briefly, 1 mL of a 1-5 MBq·mL$^{-1}$ solution of the radiolabeled compound in 0.2 M phosphate buffer (pH 7.4) was vigorously mixed with 1 mL of 1-octanol for 1 min. at room temperature using a vortex. After a settling period of 30 min, five samples of 100 µL were taken from both layers. To determine recovery, 5 samples of 100 µL were taken
from the 1-5 MBq·mL⁻¹ solution. All samples were counted for radioactivity (LKB Wallac 1282 Compugamma CS, Turku, Finland). The LogD_{oct,7.4} value was calculated according to LogD_{oct,7.4} = 10\log(A_{oct}/A_{buffer})
where \(A_{oct}\) and \(A_{buffer}\) represent average radioactivity of 5 1-octanol and 5 buffer samples, respectively.

4.2.4. Pharmacology

4.2.4.1. Animals
Adult male Wistar rats (Harlan, Horst, The Netherlands) weighing 200 to 224 g at arrival were housed in groups of five to six animals per cage with ad libitum access to food (Teklad Global 16% Protein Rodent Diet, Harlan, Madison, WI, USA) and tap water. Rats were kept in an animal room with a constant temperature of \(\sim 21\) °C and in a 12 h light/dark cycle in which lights were switched on at 8:00 am. Animal procedures were performed in accordance with Dutch laws on animal experimentation and after approval by the local animal ethics committee, study number DEC_PET12-01.

4.2.4.2. Membrane preparation, competition binding assays and data analysis
Membrane preparation, competition binding assays and data analysis were carried out according to published procedures [18].

4.2.4.3. Biodistribution and blocking studies
The biodistribution of \([^{11}\text{C}]5\), \([^{18}\text{F}]37\) and \([^{11}\text{C}]78b\) was determined in male Wistar rats without anaesthesia. Rats (N=4 per time point) received a dose of either 100 MBq \([^{11}\text{C}]5\), \([^{11}\text{C}]78b\) or 40 MBq \([^{18}\text{F}]37\) in the tail vein and were decapitated at 5, 15, 30 or 60 min. after injection. Blood, heart, lung, liver, kidney and bone (skull) were removed. In addition, several brain areas were dissected: hippocampus, striatum, hypothalamic region, cerebellum, cerebral cortex and rest brain. These tissues were counted for radioactivity with a gamma counter (LKB Wallac, Turku, Finland) and weighed. Results were expressed as percent injected dose per gram (%ID/g). For blocking studies, rats received a subcutaneous dose of 0.6 mg·kg⁻¹ MK-801 (salt weight, intraperitoneal), 15 min. prior injection of the radiotracer.

4.2.4.4. Metabolite analysis
The metabolic profile of \([^{11}\text{C}]5\), \([^{18}\text{F}]37\) and \([^{11}\text{C}]78b\) was determined in male Wistar rats without anaesthesia. Rats (N=3 per time point) received a dose of either 100 MBq \([^{11}\text{C}]5\), \([^{11}\text{C}]78b\) or 50 MBq \([^{18}\text{F}]37\) in the tail vein and were decapitated at either 15 or 45 min. after injection. A blood sample was obtained and the brain was isolated. All blood samples were collected in heparin tubes and centrifuged at 4,000 rpm and 4 °C.
for 5 min. (Hettich Universal 16, Depex B.V, The Netherlands). Plasma was separated from blood cells, 1 mL was transferred into an Eppendorf, and acetonitrile (1 mL) was added to precipitate the proteins. After centrifuging for 1 min. at 4000 rpm, the supernatant was analysed using HPLC; Dionex Ultimate 3000 HPLC system (Dionex, Breda, the Netherlands), equipped with a Phenomenex Gemini C8, 250x10.0 mm column (Phenomenex, Utrecht, the Netherlands), gradient, 0.1 M ammonium acetate/acetonitrile, 0-3 min. 10% acetonitrile, 11 min. 80% acetonitrile, 14 min. 10% acetonitrile, 4.0 mL·min⁻¹. The HPLC system was controlled using the software package Chromeleon 6.80 (Dionex, Breda, the Netherlands). Fractions of 30 s were collected and counted using a Perking Elmer 2480 single well gammacounter (Groningen, the Netherlands). Graphs were reconstructed using Excel2007® (Microsoft, Redmond, WA, USA).

Brain tissue was homogenized with an ultrasonic homogenizer (Braunsonic 1510, Kronberg, Germany) in water, under ice cooling, and subsequently centrifuged at 4,000 rpm and 4 °C for 5 min. Separated supernatants were treated as above. The recovery of the metabolite analysis was validated to be above 95%.

4.3. Results and discussion

4.3.1. Chemistry

Starting from 4, the synthesis of guanidines 77a-b, summarised in Scheme 1, was performed according to methods described previously and with comparable yields [18]. Alkylation of 77a-b was carried out by treating the phenol moiety with 1-bromo-1-fluoroethene following a method adapted from Antonsen et al [24]. At room temperature 1-bromo-1-fluoroethene is gaseous, therefore, it was condensed to 0 °C before mixing it with 1,2-dimethoxyethane and subsequent addition to the reaction mixture. After purification, 78a and 78b were obtained in 18 and 75% yields, respectively.
Scheme 1a. Synthesis of 78a-b.

\[
\begin{align*}
\text{Scheme 1a. Synthesis of 78a-b.} \\
\text{Reagents and conditions: } (i) & \text{ cyanogen bromide, diethyl ether, } 0 \degree \text{C} \rightarrow \text{rt}, 17 \text{ h}; (ii) \text{ methyl iodide, potassium carbonate, } N,N\text{-dimethylformamide, rt, 3 h; (iii) } 2\text{-chloro-5-(methylthio)aniline hydrochloride, chlorobenzene, } 165 \degree \text{C, 6 h; (iv) sodium hydride, sodium iodide, 1-bromo-1-fluoroethene, 1,2-dimethoxyethane / } N,N\text{-dimethylformamide, } 60 \degree \text{C, 24 h.}
\end{align*}
\]

### 4.3.2. Affinity of 78b

Table 1 shows the affinities of 5, 37 and 78b (tested as fumaric salts) in nM for the ion channel of the NMDAr against 5 nM of \[^3\text{H}\]\text{MK-801}. Remarkably, affinities of all compounds were in the same range. This again indicates that small structural alterations in the methoxy moiety are tolerated by the NMDAr.

The affinity of 5 was previously reported as 5.2 and 21.7 nM respectively [8,18]. The discrepancy in affinity between these two studies is most likely due to differences in the method of determining the affinity towards the NMDAr. In the present study compounds 5, 37 and 78b were tested under the same conditions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵢ (nM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ᵇ</td>
<td>21.7 ± 7.5</td>
</tr>
<tr>
<td>37ᵇ</td>
<td>18.4 ± 8.1</td>
</tr>
<tr>
<td>78b</td>
<td>18.6 ± 7.1</td>
</tr>
</tbody>
</table>

ᵃKᵢ measured against 5 nM \[^3\text{H}\]\text{MK-801 and presented as mean ± SD of 4 independent determinations, each conducted in triplicate.}

ᵇKᵢ values taken from Klein \textit{et al} [18].
4.3.3. Radiochemistry

Compound 78a was radiolabeled with $[^{11}\text{C}]\text{CH}_3\text{I}$ on the N-methyl position of the guanidine next to the vinyloxyphenyl moiety at 60 °C in 3 minutes using NaOH as base in DMSO. $[^{11}\text{C}]78b$ (Scheme 2) was prepared in a one-pot synthetic procedure in 20 ± 6 % (decay-corrected, calculated from $[^{11}\text{C}]\text{CH}_3\text{I}$) yield, providing 4.1 ± 1.4 GBq (N=10) of formulated product at the end of synthesis with a radiochemical purity of >99% and with a specific radioactivity of 112 ± 27 GBq/μmol (N=4). During synthesis of $[^{11}\text{C}]78b$ a side product, 78a $[^{11}\text{C}]$methylated at the other guanidine N, was formed. This side product was easily removed using preparative HPLC. Using these reaction conditions a sufficient amount of $[^{11}\text{C}]78b$ was obtained to perform the preclinical experiments. Further optimization was not performed.

Scheme 2a. Synthesis of $[^{11}\text{C}]78b$.

\[ \text{Reagents and conditions: (i) } [^{11}\text{C}]\text{CH}_3\text{I}, \text{NaOH, DMSO, 60 °C, 3 min.} \]

In contrast to $[^{11}\text{C}]78b$, ligands $[^{11}\text{C}]5$ and $[^{18}\text{F}]37$ were radiolabeled at the phenolic moiety. Currently there is no method known for radiolabeling the fluoro-vinyloxy moiety with either carbon-11 or fluorine-18. Both $[^{11}\text{C}]5$ and $[^{18}\text{F}]37$ could be radiolabeled with carbon-11 in the same position as in $[^{11}\text{C}]78b$, but it was decided to keep both ligands original as published before. Compound 5 and 37 were radiolabeled as described previously in sufficient yields and high radiochemical purity and specific activity [10,18].

4.3.4. Lipophilicity of $[^{11}\text{C}]78b$

The lipophilicity of compound $[^{11}\text{C}]78b$ is shown in Table 2 and was determined as the LogD$_{7.4}$ in octanol BUFFER. Table 2 compares the LogD$_{7.4}$ values of 5, 37 and 78b. The lipophilicity of ligand 78b is well within the borders of 2.0 – 3.5 stated by Pike indicating an optimal passive brain entry in vivo [25].

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogD$_{oct,7.4}$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{11}\text{C}]5^b$</td>
<td>1.72 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>$[^{18}\text{F}]37^b$</td>
<td>1.45 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>$[^{11}\text{C}]78b$</td>
<td>2.62 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

\[^a\text{N=3, presented as mean } \pm \text{ SD.} \quad ^b\text{Values taken from Klein et al [18].}\]
4.3.5. Ex vivo biodistribution of $[^{11}C]78b$

Ex vivo biodistribution and regional brain uptake of $[^{11}C]78b$ were determined in non-anaesthetized male Wistar rats, to avoid any confounding effect of anaesthesia on NMDAr binding.

Total brain uptake of $[^{11}C]78b$ at 5, 15, 30 and 60 min. after injection was $0.76 \pm 0.08$, $0.70 \pm 0.05$, $0.40 \pm 0.04$ and $0.28 \pm 0.01 \%$ID/g, respectively. Figure 2 shows regional and total brain uptake as well as organ uptake. $[^{11}C]78b$ showed uptake across all dissected brain areas, with initial higher uptake in prefrontal cortex and the lowest in cerebellum. Organ uptake of $[^{11}C]78b$ was highest in the lungs followed by kidneys and liver.

![Figure 2. Mean (± SEM) uptake of $[^{11}C]8b$ in CNS (left) and various organs (right) expressed in %ID/g (N=4 for each time point).](image)

4.3.6. Blocking studies of $[^{11}C]78b$

MK-801 (0.6 mg·kg$^{-1}$) was used to determine the specificity of uptake of ligand $[^{11}C]78b$. The blocking dose was chosen to be two-to-three times the in vivo $ED_{50}$ of MK-801 for NMDArs [26,27]. MK-801 was administered 15 min prior to injection of the radiotracer, as previous studies have shown that $T_{max}$ of MK-801 is obtained 10 to 30 minutes after administration [27]. Figure 3 shows the tracer biodistribution at 15 min. with and without MK-801 pre-treatment. Total brain uptake at 15 min was $0.80 \pm 0.05$ before and $0.66 \pm 0.03 \%$ID/g ($P < 0.05$, unpaired t test with Welch’s correction, N=4, mean ± SEM) after pre-treatment. This overall decrease represents an 18% reduction from control, which is well within the range of maximum specific accumulation that can be achieved for ion-channel blockers using this type of in vivo assay [26,29]. Individual t-tests, conducted for each brain area separately, showed no region-specific differences in the uptake of $[^{11}C]78b$ after MK-801 administration.
4.3.7. Metabolite analysis of $[^{11}\text{C}]5$, $[^{18}\text{F}]37$ and $[^{11}\text{C}]78b$

When using PET as quantitative method to image the human brain, it is important to consider metabolism of a radioligand in vivo, as it is not possible to distinguish between signals from radiolabeled metabolites and parent compound. Firstly, radiolabeled metabolites should not cross the blood brain barrier as it may hamper interpretation of measured signals from the brain. Secondly, the speed of metabolism is an important factor. The lower the amount of parent compound, the lower the signal from the brain, leading to a decreased noise/signal ratio.

Metabolites were determined in non-anesthetized male Wistar rats. Figure 4 summarises parent compound and labelled metabolite fractions for $[^{11}\text{C}]5$, $[^{18}\text{F}]37$ and $[^{11}\text{C}]78b$ in plasma and brain. See supplementary data for representative chromatograms. The first radioactivity peak observed with HPLC was identified as the polar fraction, most likely representing single carbon molecules such as formaldehyde, formic acid or carbon dioxide. The last peak was identified as the parent compound. Apart from these polar metabolite and parent fractions, multiple non-polar peaks were observed, i.e. 2, 3 and 2 peaks in plasma, and 3, 3 and 2 peaks in brain for $[^{11}\text{C}]5$, $[^{18}\text{F}]37$ and $[^{11}\text{C}]78b$, respectively. For each compound, the retention times of metabolites and parent compound observed in plasma were identical to the retention times observed in brain.
Figure 4. Distribution of radiolabeled polar metabolites, radiolabeled non-polar radiolabeled metabolites and parent compound fractions in male Wistar rats of $[^{11}C]5$, $[^{18}F]37$ and $[^{11}C]78b$ in plasma (left side) and brain (right side) at 15 and 45 min. (N=3 ± SD).

The amount of parent compound in plasma and brain at 15 and 45 min. is summarised in Figure 5. In plasma, the parent fraction was 42 – 47% at 15 min. and 26 – 31% at 45 min. for all three radiotracers. Although no significant difference between the radiotracers could be observed, a slight trend between the radiotracers is visible. This trend is possibly related to the degree of substitution of hydrogen atoms in the methoxy moiety.
After 15 min, for all three ligands, 91 – 94% of radioactivity in the brain was due to parent compound. After 45 min the amount of parent compound of \([^{11}\text{C}]5\) was reduced to 78%. For both \([^{18}\text{F}]37\) (90%) and \([^{11}\text{C}]78b\) (87%) the amount of parent compound was significant higher than for \([^{11}\text{C}]5\).

Figure 5. Parent fraction of \([^{11}\text{C}]5\), \([^{18}\text{F}]37\) and \([^{11}\text{C}]78b\) in plasma (left side) and brain (right side) of male Wistar rats at 15 and 45 min. (** P<0.01, *** P<0.001).

Multiple chemical moieties of \([^{11}\text{C}]5\), \([^{18}\text{F}]37\) and \([^{11}\text{C}]78b\) are prone to metabolism, e.g. by oxidation or demethylation. In a recent \([^{18}\text{F}]\text{GE-179}\) study it is shown that one of the metabolites was likely to be a sulfoxide derivative \([17]\). The amount of metabolites formed by sulphur oxidation could be assumed to be the same for all three compounds. Previously, a structure-activity relationship of CNS-5161 and its sulfoxide derivatives has been reported \([28]\). This study showed a 455 fold decrease in affinity towards the NMDAr when the sulphur was oxidized. Assuming that the affinity will drop dramatically when the sulphur is oxidized, this radiolabeled metabolite would not interfere with the binding of \([^{11}\text{C}]5\), \([^{18}\text{F}]37\) and \([^{11}\text{C}]78b\), respectively.

N-demethylation is primarily caused by the cytochrome P450 enzyme system \([30]\). Ligand \([^{11}\text{C}]78b\), which is radiolabeled at the guanidine N-methyl, shows only 1% of polar metabolites after 45 minutes. Likely, this process does not contribute much to the amount of radiolabeled metabolites measured in the brain. In addition, previous structure-activity relationship studies have shown that omitting the N-methyl in similar compounds decreased their affinity towards the NMDAr by 6 to 20 fold \([18]\).

It is known that metabolically labile sites can be made less vulnerable to metabolism by substitution with fluorine \([31]\). Compared with \([^{11}\text{C}]5\), \([^{18}\text{F}]37\) indeed showed a slightly lower rate of metabolism, but at both 15 and 45 min. no significant difference was observed between the parent fractions of \([^{11}\text{C}]5\) and \([^{18}\text{F}]37\) in plasma.

By further substitution of the fluoromethoxy moiety with a vinyl group (\([^{11}\text{C}]78b\)), it was anticipated that the rate of metabolism would be further decreased, as this should prevent oxidation by the cytochrome P450 system. Again, a slight decrease in
rate of metabolism could be observed, but again the parent fraction in plasma of [\textsuperscript{11}C]\textbf{78b} was not significantly higher than that of [\textsuperscript{18}F]\textbf{37} at either 15 or 45 min.

To summarize, at 15 min. the fraction of intact parent compound of [\textsuperscript{11}C]\textbf{5}, [\textsuperscript{18}F]\textbf{37} and [\textsuperscript{11}C]\textbf{78b} in the brain was in the same range (more than 90%). At 45 min, the parent fraction still was 90\% for [\textsuperscript{18}F]\textbf{37}, followed by [\textsuperscript{11}C]\textbf{78b} (87\%) and [\textsuperscript{11}C]\textbf{5} (78\%).

Based on results presented in this study, both [\textsuperscript{18}F]\textbf{37} and [\textsuperscript{11}C]\textbf{78b} might be better PET tracers for imaging the NMDAr than [\textsuperscript{11}C]\textbf{5}. Both radiotracers showed a slightly improved affinity to the ion channel of the NMDAr and a small increase in metabolic stability. Compound [\textsuperscript{11}C]\textbf{78b} has a favourable lipophilicity over compounds [\textsuperscript{11}C]\textbf{5} and [\textsuperscript{18}F]\textbf{37}. In addition, [\textsuperscript{11}C]\textbf{78b} showed reduced uptake following pre-treatment with the ion channel blocker MK-801. On the other hand compound [\textsuperscript{18}F]\textbf{37} showed less radioactive polar metabolites in the brain. However, further studies are needed in non-human primates, but preferably in humans, as anaesthesia could interfere with NMDAr binding [32].

4.4. Acknowledgments

The authors would like to thank Rolph van Kooij for his assistance in the radiosynthesis and the BV Cyclotron VU for providing [\textsuperscript{11}C]CO\textsubscript{2}.

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4.5. Supplementary data

Figure S1. Representative HPLC chromatograms of metabolite analysis of $[^{11}\text{C}]5$, $[^{18}\text{F}]37$ and $[^{11}\text{C}]78\text{b}$ in rat of plasma and brain.
4.6 References


Chapter 5.

Preclinical evaluation of $[^{18}\text{F}]$PK-209, a new PET ligand for imaging the ion-channel site of NMDA receptors

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Abstract

Introduction: The present study was designed to assess whether $[^{18}F]$PK-209 (3-(2-chloro-5-(methylthio)phenyl)-1-(3-($[^{18}F]$fluoromethoxy)phenyl)-1-methylguanidine) is a suitable ligand for imaging the ion-channel site of $N$-methyl-D-aspartate receptors (NMDArs) using positron emission tomography (PET).

Methods: Dynamic PET scans were acquired from male rhesus monkeys over 120 min, at baseline and after the acute administration of dizocilpine (MK-801, 0.3 mg/kg; n=3/condition). Continuous and discrete arterial blood samples were manually obtained, to generate metabolite-corrected input functions. Parametric volume-of-distribution ($V_T$) images were obtained using Logan analysis. The selectivity profile of PK-209 was assessed in vitro, on a broad screen of 79 targets.

Results: PK-209 was at least 50-fold more selective for NMDArs over all other targets examined. At baseline, prolonged retention of radioactivity was observed in NMDAr-rich cortical regions relative to the cerebellum. Pretreatment with MK-801 reduced the $V_T$ of $[^{18}F]$PK-209 compared with baseline in two of three subjects. The rate of radioligand metabolism was high, both at baseline and after MK-801 administration.

Conclusions: PK-209 targets the intrachannel site with high selectivity. Imaging of the NMDAr is feasible with $[^{18}F]$PK-209, despite its fast metabolism. Further in vivo evaluation in humans is warranted.
5.1. Introduction

N-methyl-D-aspartate receptors (NMDArs) are glutamate-gated cation channels with key physiological roles in the developing and adult brain. Their timely activation is required for synaptic maturation and plasticity, underlying mental processes as essential for life as memory and learning [1]. The aberrant activation of NMDArs, however, can have devastating effects on neuronal survival and physiological brain function. NMDAr overactivation is known to promote cell death by excitotoxicity [2], a process that has been implicated in the pathogenic mechanisms of ischaemia, epilepsy and chronic neurodegenerative disorder, including Alzheimer’s, Parkinson’s and Huntington’s diseases [3]. NMDAr hypofunction, on the other hand, has been linked to the pathophysiology of schizophrenia and autism spectrum disorder [4], and may be related with increased risk for drug abuse [5]. Because of the substantial roles of NMDArs in health and disease, there is considerable interest in developing an imaging tool to assess the functional state of the NMDAr in vivo, both for diagnostic and research purposes.

The activation of NMDArs is controlled by multiple endogenous ligands, which tightly regulate the probability of ion-channel opening by binding to distinct sites on the receptor [6]. There are at least six different binding sites on the NMDArs, including recognition sites for the agonist glutamate, the co-agonists glycine and serine, as well as for ligands that modulate receptor function, such as polyamines, metal ions and protons. Of the multiple binding sites, the one that is located within the pore of the ion-channel is an attractive target for examining the functional state of NMDArs. Due to its unique physical location, the ion-channel site is primarily accessible when the NMDAr is in an open conformation, i.e. activated by glutamate and glycine [7]. Thus, the in vivo uptake of radiotracers targeting the intrachannel site is likely to be proportional to the amount of activated NMDArs, and could be used to determine their functional state and brain regional distribution [8].

Although there have been several attempts to label the ion-channel site using Positron Emission Tomography (PET), the majority of efforts has been confounded by poor radiotracer selectivity and affinity, low brain entrance, rapid radioligand metabolism, and/or inability to establish specific NMDAr targeting in vivo [9-10]. We have previously described the synthesis, radiosynthesis, in vitro and ex vivo evaluation of a series of N,N’-diaryl-N-methylguanidines, targeting the NMDAr ion-channel site [11]. In that series, [18F]PK-209 ([18F]37, Figure 1) was the most promising candidate ligand for imaging NMDArs. The compound had an apparent affinity value of 18 nM against [3H]MK-801 (Kᵢ) and a distribution coefficient value of 1.45 (LogDoct,7.4), indicating high affinity for the NMDAr and moderate lipophilicity, respectively. In
biodistribution experiments in mice, the forebrain-to-cerebellum ratio of radioactivity uptake after the administration of $[^{18}\text{F}]$PK-209 was in excess of 1.7, indicating accumulation of the tracer in NMDAr-rich brain regions. Importantly, ex vivo autoradiography showed that the brain uptake of $[^{18}\text{F}]$PK-209 was reduced by up to 30% from control levels after the acute administration of dizocilpine (MK-801; 0.6 mg/kg, intraperitoneally), indicating selectivity of the radioligand for the NMDAr ion-channel site. In the present study, we report on a comprehensive pharmacological selectivity profile for PK-209, and examine whether selective labeling and quantification of the NMDA receptor ion-channel is feasible in the nonhuman primate brain using $[^{18}\text{F}]$PK-209 PET.

![Chemical structure of $[^{18}\text{F}]$PK-209](image)

Figure 1. Chemical structure of $[^{18}\text{F}]$PK-209 ($[^{18}\text{F}]{\text{37}}$, 3-(2-chloro-5-(methylthio)phenyl)-1-(3-([$^{18}\text{F}$]fluoromethoxy)phenyl)-1-methylguanidine).

### 5.2. Materials and Methods

#### 5.2.1. Selectivity profile of PK-209

The pharmacological selectivity of PK-209 was evaluated at Cerep (Poitiers, France). A single concentration of PK-209 (10 μM) was initially screened on a panel of 79 targets, comprising neurotransmitter receptors and transporters, and ion-channels (Table S1, Supplementary data). A percent (%) inhibition of control specific binding, obtained in the presence of 10 μM PK-209, was calculated for each target. The affinity of PK-209 for targets showing ≥50% inhibition of control specific binding in the primary screen was subsequently derived from full concentration-response inhibition curves, which were obtained using 8 concentrations of PK-209. Hill coefficients (nH) and IC$_{50}$ values from the competition binding experiments were derived by non-linear regression analysis. IC$_{50}$ values were converted to inhibition constants (Ki values) using the Cheng and Prusoff equation for binding assays: $K_i=IC_{50}/(1+L/K_D)$, where L is radioligand concentration in the assay and $K_D$ the affinity of the radioligand for its corresponding target. The $K_D$ values of the reference radioligands were obtained in saturation experiments, which were run in parallel to the competition binding studies. Details of the Cerep selectivity assays are available from www.cerep.fr.
5.2.2. Radiotracer preparation

The precursor synthesis [12] and radiolabeling procedures [13] for $^{18}$F]PK-209 have been described previously. The radiotracer was formulated in a phosphate-buffered saline solution containing 8.6% ethanol, and administered by intravenous injection of ≤10 mL. For baseline studies, injected activity was 138.8±30.4 MBq and specific activity 135.8±15.4 GBq/µmol, corrected to scan acquisition time. For blocking studies, injected activity and specific activity at scan acquisition time were 124.7±19.5 MBq and 97.2±24.4 GBq/µmol, respectively. In all cases, synthesis time including HPLC purification was ≈90 min, and radiochemical purity of the final product >98%.

5.2.3. Subjects, anaesthesia and treatment

All experimental procedures complied with European Commission Directive 2010/63/EU, regulating animal research, and were approved by the independent Animal Experimental Committee of the Biomedical Primate Research Centre in Rijswijk, the Netherlands (BPRC; DEC#717BPRC).

Three adult male rhesus monkeys (Macaca mulatta) were included in the study, aged 17-19 years and weighing 7.6-13.5 kg. Monkeys were group-housed at the BPRC, in an enriched, temperature-controlled environment (20-22°C), under a 12 h light/dark circadian cycle. The animals were maintained on a standard monkey pellet diet (Ssniff®, Soest, Germany) supplemented with vegetable and fresh fruit, and were fasted for a period of 16 h prior to anaesthesia. Drinking water was available ad libitum. All animals had received a comprehensive physical, haematological and biochemical examination prior to study initiation and remained under constant veterinary supervision throughout the experiments.

On the day of each baseline scan, monkeys were transported from the BPRC in Rijswijk to the Radionuclide Center in Amsterdam (RNC, Amsterdam, the Netherlands). The animals were trained to voluntary enter their transit cages, and no anaesthesia was used during the 40 min transportation period. At the RNC, monkeys were sedated with an injection of medetomidine hydrochloride (60 μg/kg; Sedastart 1 mg/mL, AST Farma BV, Oudewater, the Netherlands) and midazolam (0.3 mg/kg; Midazolam Actavis 5 mg/mL; Actavis BV, Baarn, the Netherlands), which were administered intramuscularly (IM). Immobilized animals were brought into the imaging facilities, and placed on a warm Harvard blanket in order to maintain normothermia (Homeothermic Blanket 60x90cm, Harvard Apparatus GmbH, March-Hugstetten, Germany). The cephalic veins of both arms were catheterized with a Vasofix® Braunüle® catheter (B. Braun Melsungen AG, Germany). The left cephalic line
was used for the induction and maintenance of propofol anesthesia, which was
delivered at a rate of 0.2 mg/kg/min (PropoVet Multidose 10 mg/mL, Fresenius Kabi
AB, Uppsala, Sweden). The right cephalic line was used to infuse NaCl/glucose, at a
rate of 1 mL/kg/h (0.45% Sodium chloride & 2.5% Glucose 500ML, Baxter BV,
Utrecht, the Netherlands). For endotracheal intubation, xylocaine 10% was sprayed
into the larynx and trachea (AstraZeneca BV, Zoetermeer, the Netherlands). The
monkeys were subsequently positioned in the scanner in the supine position, with
their head fixed at the appropriate orientation within the field of view, using custom-
built face masks. Haemoglobin oxygen saturation levels (SpO2) and heart rate were
continuously measured with a Mindray monitor (PM-8000, GmbH, Hamburg,
Germany). Body temperature was continuously monitored with a rectal probe (PM-
8000, GmbH, Hamburg, Germany). At the end of each scan, and following recovery
from immobilization, animals were transported from the RNC in Amsterdam to the
BPRC in Rijswijk. Full recovery from anaesthesia was defined as the animal’s ability to
be safely reunited with its home-cage companions, after walking and climbing
confidently in the transit cage [14].

Blocking scans were performed one week after baseline for subjects 1 and 2, and
two weeks after baseline for subject 3, according to the above described protocol. (+)-
MK-801 hydrogen maleate was used to block NMDArs (Sigma Aldrich, Zwijndrecht,
the Netherlands). The drug was administered via the right cephalic vein, 30 min prior
to radiotracer injection, at a dose of 0.3 mg/kg (free-base weight). The blocking dose
and timing of MK-801 administration were based on glutamate microdialysis studies
in the rhesus monkey [15], and were chosen to avoid alterations in extracellular levels
of glutamate following NMDAr antagonism.

5.2.4. Image acquisition

Magnetic Resonance Imaging (MRI) data were acquired prior to the PET studies, using
a 3 Tesla Siemens Trio scanner (Siemens, Erlangen, Germany), and a standard T1-
weighted 3D MPRAGE sequence (TR 2300 ms, TI 1100 ms, TE 3.93 ms, 192 sagittal
slices, field of view 256 mm). All images were reconstructed into a matrix of 192 ×
256 × 256 voxels, with a voxel size of 1.0×1.0×1.0 mm.

PET measurements were performed on a Siemens ECAT HRRT scanner, operated
in 3D mode (CTI/Siemens, Knoxville, TN, USA). A detailed description of the HRRT
scanner, its reconstruction software and performance has been reported previously
[16]. For attenuation and scatter correction, each PET scan was preceded by a 6 min
transmission scan, which was performed using a 740 MBq $^{137}$Cs (662 keV) rotating
point source. Dynamic PET scans were acquired over 120 min (frame end time),
starting simultaneously with a bolus injection of \([^{18}\text{F}]\)PK-209 into the right cephalic vein. Data were stored in 64-bit list mode format and subsequently binned into 20 time frames. Frame sequence definition was 6x10s, 2x30s, 3x60s, 2x150s, 2x300s, 2x600s, 2x1200s, and 1x2400s. Reconstruction was performed using the ordered subsets weighted, least-squares algorithm. All data were normalized and corrected for scatter, random coincidences, attenuation, decay and dead time. All images were reconstructed into a matrix of \(256 \times 256 \times 207\) voxels, with a voxel size of \(1.218 \times 1.218 \times 1.218\) mm.

5.2.5. Blood sampling and metabolite analysis

Blood was manually sampled from the femoral artery using a 20G Vacuette (Vacuette 20G x 1 ½”; Greiner Bio-One GmbH, Kremsmünster, Austria), collected into heparin tubes (Greiner Bio-One GmbH, Kremsmünster, Austria), and mixed by inversion. Sampling began simultaneously with an injection of \([^{18}\text{F}]\)PK-209, and continued for a period of 5 min, at 5-10 s intervals. Radioactivity in 100 μL aliquots of whole-blood was directly measured in a Wallac 2480 Wizard² Automatic Gamma Counter (PerkinElmer, Groningen, the Netherlands). Discrete blood samples were collected at 5, 10, 15, 30, 60, 90 and 120 min post-injection, to measure plasma and whole-blood ratios, calibrate the whole-blood data, and determine metabolite fractions.

For metabolite analysis, blood samples were centrifuged at 4,000 rpm for 5 min at 4°C, in a Hettich Universal 16 table centrifuge (Hettich Benelux BV, Geldermalsen, the Netherlands). Plasma was separated from blood cells and loaded onto an activated tC2 Sep-Pak cartridge (Waters, Etten-Leur, the Netherlands), which was washed with 3 mL of demineralised (demi) water. The eluate was defined as the polar radiolabeled metabolite fraction. The tC2 Sep-Pak was then washed with 2 mL of methanol and 1 mL of demi water. This eluate was defined as the non-polar fraction, and analyzed using reverse phase HPLC [Dionex Ultimate 3000 HPLC system, equipped with a Phenomenex Gemini C18 5μm 250 x 10.0 mm column, using a gradient of 50 mM NH₄H₂PO₄ / acetonitrile (80/20 to 30/70, v/v) in 12.5 minutes at a flow rate of 3.0 mL/min]. Radioactivity in polar and non-polar fractions was measured off-line in a Wallac 2480 Wizard² Automatic Gamma Counter (PerkinElmer, Groningen, the Netherlands).
5.2.6. Image processing

MRI and PET images were coregistered using the software package VINCI [17]. Individualized, whole-brain masks were manually drawn for each averaged PET image (frames 5-16) and for the MRI image of each subject, to ensure coregistration based on brain voxels only. Regions-of-interest (ROIs) were automatically delineated onto coregistered MRI scans using the INIA19 template for the rhesus monkey brain [18]. The following regions were defined for the left and right hemispheres: frontal lobe, temporal lobe, occipital lobe, striatum (caudate and putamen), and cerebellum. Whole-brain gray matter voxels were also included. ROIs were projected onto the complete dynamic PET images to extract the corresponding time-activity curves, using in-house software written in MATLAB® (R2007b, The MathWorks, Natick, MA, USA). The arterial plasma input function was derived from continuous and discrete blood samples, which were corrected for parent fraction, whole blood/plasma ratio and delay. The 1 minus polar fraction was used as a surrogate of unmetabolized [18F]PK-209. Parametric volume-of-distribution (VT) images were used to evaluate the VT of [18F]PK-209 at baseline conditions and following the administration of MK-801. Images were generated using plasma-input Logan analysis [19].

5.2.7. Data analysis and statistics

Radioactivity concentration was expressed as percentage of injected dose per cm³ of brain tissue (%ID/cm³). Area under the time-activity curves (AUC0-100.5; frame midpoint times) was calculated as a simplified measurement of accumulated brain uptake of [18F]PK-209-derived radioactivity. The between-subjects coefficient of variation (CV) was calculated as the ratio of standard deviation to mean VT values. The systemic clearance of [18F]PK-209 was calculated as injected activity, divided by area under the arterial plasma concentration curve (extrapolated to infinity). Non-parametric statistics were used to analyse all data sets. Friedman tests ($\chi^2$) for the factors brain region and treatment were used to compare mean VT values of [18F]PK-209 between baseline and blocking conditions in individual brain areas. Wilcoxon matched-pairs tests (Z) were used to compare the overall VT of [18F]PK-209 before and after the administration of MK-801, at the within-subject level. Where applicable, results are presented as mean ± standard error of the mean (SEM).
5.3. Results

5.3.1. In vitro pharmacological selectivity profile of PK-209

At a concentration of 10 µM, PK-209 showed no significant binding to 70 of the 79 targets tested. Affinity values for targets showing ≥50% inhibition of control specific binding in the primary screen are listed in Table 1. PK-209 exhibited >50-fold selectivity for the ion-channel of NMDAr over all other targets examined.

Table 1. Affinity values of PK-209 (IC₅₀/Kᵢ) and slope of the inhibition curves (Hill coefficient, nH) for targets showing ≥50% inhibition of control specific binding in the primary screen assays.

<table>
<thead>
<tr>
<th>Target</th>
<th>IC₅₀ (M)ᵃ</th>
<th>Kᵢ (M)ᵇ</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Adrenergic alpha 1A</td>
<td>6.2·10⁻⁶</td>
<td>3.1·10⁻⁶</td>
<td>1.1</td>
</tr>
<tr>
<td>2 Muscarinic M1</td>
<td>5.3·10⁻⁶</td>
<td>4.6·10⁻⁶</td>
<td>1.0</td>
</tr>
<tr>
<td>3 Muscarinic M2</td>
<td>4.2·10⁻⁶</td>
<td>2.9·10⁻⁶</td>
<td>0.8</td>
</tr>
<tr>
<td>4 Opioid κ (KOP)</td>
<td>7.5·10⁻⁶</td>
<td>5.0·10⁻⁶</td>
<td>1.0</td>
</tr>
<tr>
<td>5 Opioid μ (MOP)</td>
<td>2.8·10⁻⁶</td>
<td>1.2·10⁻⁶</td>
<td>0.8</td>
</tr>
<tr>
<td>6 NMDA (PCP site)</td>
<td>4.0·10⁻⁸</td>
<td>2.2·10⁻⁸</td>
<td>1.5</td>
</tr>
<tr>
<td>7 sigma (1 and 2)</td>
<td>2.2·10⁻⁶</td>
<td>1.8·10⁻⁸</td>
<td>0.9</td>
</tr>
<tr>
<td>8 Ca²⁺ channel (L verapamil site)</td>
<td>4.1·10⁻⁶</td>
<td>2.0·10⁻⁶</td>
<td>0.7</td>
</tr>
<tr>
<td>9 Na⁺ channel (site 2)</td>
<td>5.2·10⁻⁶</td>
<td>4.6·10⁻⁶</td>
<td>1.0</td>
</tr>
</tbody>
</table>

ᵃIC₅₀ is the concentration of PK-209 inhibiting 50% of reference radioligand binding to the target of interest, and was obtained in assay conditions as described in the Cerep catalogue (Cerep, Poitiers, France).

ᵇKᵢ values were obtained by transforming IC₅₀ values according to the Cheng & Prusoff equation. For the transformation, KD values of the reference radioligands were measured in parallel experiments.

5.3.2. Physiological response to anaesthesia

The sedative effect of medetomidine/midazolam occurred without excitation. Immobilization was induced within 10-15 min of drug administration for all subjects. Animals were breathing spontaneously under propofol anaesthesia, at a stable rate of 30-40 breaths/min. Percent oxygen saturation (≥95%) and heart rate (120-160 beats/min) were not suppressed during the baseline or blocking scans. Body temperature decreased gradually during both baseline and blocking experiments, from 37.5-38.0 °C to 34.5-35.5°C. Full recovery from anaesthesia occurred within 4 h of propofol cessation on the day of the baseline scans. Return to pre-anaesthetic condition was prolonged to >8 h by the administration of MK-801. Recovery from anaesthesia was uneventful for subjects 1 and 2. Subject 3 showed symptoms of general discomfort, lacrimation, agitation, and loss of appetite following its baseline scan. The animal responded to three days of treatment with buprenorphine (0.02
mg/kg, twice daily, IM) and meloxicam (0.10 mg/kg, once daily, per os), and was scanned healthy 2 weeks after baseline.

5.3.3. Metabolism of $^{18}$F]PK-209

Figure 2 shows mean fractions in the plasma of unchanged $^{18}$F]PK-209 and its metabolites as a function of time. 10 min after the administration of $^{18}$F]PK-209, 60±4% of total radioactivity in plasma was due to polar metabolites. HPLC analysis of the non-polar fraction at the 10 min time-point demonstrated that unchanged $^{18}$F]PK-209 and 1 major metabolite accounted for 30±6% and 8±3% of total plasma radioactivity, respectively. Retention times were 12.6 min for $^{18}$F]PK-209 and 10.1 min for the more-polar metabolite. The recovery of radioactivity off the HPLC was >97% over a 15 min run. The mean percentage of plasma radioactivity attributable to non-metabolised tracer throughout the 2 h studies was 19.2±0.5%, while polar and non-polar metabolites of $^{18}$F]PK-209 accounted for 75.0±0.5% and 5.7±0.1% of the overall plasma radioactivity, respectively. No radioactivity uptake was observed in the jaw and skull in the dynamic PET images (Figure S1 Supplementary data). Wilcoxon matched-pairs tests were used to compare parent and metabolite fractions of radioactivity between baseline and blocking scans at individual time-points. There was no effect of MK-801 administration on the metabolism of $^{18}$F]PK-209 ($P>0.05$ for each time-point).

Figure 2. Fractions of the plasma $^{18}$F activity of unchanged PK-209 and its metabolites as a function of time. Blood samples were collected at 5, 10, 15, 30, 60, 90 and 120 min after the administration of $^{18}$F]PK-209. The mean percentage of plasma radioactivity attributable to parent $^{18}$F]PK-209, polar, and non-polar metabolites during the 2 h scans was 19.2±0.5%, 75.0±0.5%, and 5.7±0.1%, respectively. Due to rapid metabolism, the 1 minus polar fraction was used as a surrogate of unchanged tracer. Results at each time-point are presented as the mean±SEM of the parent, polar and non-polar radioactivity fractions from 3 monkeys/scanning condition.
5.3.4. Pharmacokinetics of $[^{18}F]$PK-209

The parent plasma input function peaked at 37.8±4.2 s post-injection, and decreased to 10% of its peak value within 3 min (Figure 3). The mean area under the input function curve was 20.6±6.6 MBq/mL·min and 18.3±4.6 MBq/mL·min for the baseline and blocking conditions, respectively ($Z=0.0$, $P>0.05$). Under both conditions, plasma to whole-blood radioactivity ratios ranged from 1.0±0.1 at 5 min post-injection, to 1.2±0.0 at 30 min, remaining stable thereafter until completion of the 2 h scans. The terminal plasma clearance of $[^{18}F]$PK-209 was 6.6±0.5 mL/min at baseline, and did not change after treatment with MK-801 (6.8±0.4 mL/min; $Z=0.0$, $P>0.05$).

Figure 3. Mean decay- and metabolite-corrected $[^{18}F]$PK-209 plasma input function. Blood from the femoral artery was manually sampled at 5-10 s intervals for a period of 5 min, beginning simultaneously with an injection of $[^{18}F]$PK-209. The manual blood time-activity curve was calibrated using the radioactivity concentrations in the discrete blood samples. Parent plasma input functions were then calculated for each subject by multiplying the calibrated manual curve with the fits to the plasma/whole-blood radioactivity ratios and parent fractions. The plasma $[^{18}F]$PK-209 input function peaked at 37.8±4.2 s post-injection, and decreased to 10% of its peak value within 3 min, both under baseline and blocking conditions.

5.3.5. Regional distribution of radioactivity and time-activity curves (TACs), at baseline and after MK-801 treatment

Figure 4 shows TACs of all ROIs examined under baseline and blocking conditions. Uptake in the whole-brain peaked at 16.3±1.7 min during the baseline scans, and was equivalent to 0.029±0.002 %ID/cm$^3$. The rank order of peak radioactivity concentration (%ID/cm$^3$) was striatum (0.036±0.004) = cerebellum (0.036±0.005) > occipital (0.033±0.004), temporal (0.029±0.001), and frontal lobes (0.028±0.001). The washout of $[^{18}F]$PK-209-derived radioactivity varied between brain regions. Concentrations at 100.5 min declined to 37.2±5.0% of the peak value in the cerebellum, to 45.3±5.3% in the striatum, and to 61.7±6.5% in the frontal lobe.
Figure 4. Decay-corrected time-activity curves of [18F]PK-209 in different brain regions, at baseline and after treatment with MK-801. Regions-of-interest (ROIs) were automatically defined using the INIA19 template for the rhesus monkey brain. ROIs were projected onto the complete dynamic PET images in order to extract time-activity curves. Radioactivity concentrations were calculated separately in areas of the left and right hemisphere, and expressed as percentage of injected dose per cm³ of tissue (%ID/cm³). Individual values from left and right ROIs were averaged within each subject, before calculating mean±SEM values of radioactivity concentration at the mid time-point of each frame. The rank order of peak radioactivity concentration was striatum and cerebellum > occipital, temporal, and frontal lobes, under both baseline and blocking conditions. At baseline, the washout of [18F]PK-209-derived radioactivity was slower from NMDAr-rich brain regions relative to the cerebellum. Pretreatment with MK-801 (0.3 mg/kg), 30 min before the administration of [18F]PK-209 reduced the mean area under the time-activity curve from 2.31±0.07 at baseline to 1.97±0.04 %ID/cm³·min. Peak uptake values were observed earlier than baseline during the blocking scans.
The mean area under the TACs (AUC0-100.5) of all regions analysed was 2.31±0.07 %ID/cm³·min for the baseline condition.

Treatment with MK-801 30 min before the administration of [18F]PK-209 decreased the mean AUC0-100.5 to 1.97±0.04 %ID/cm³·min, representing a reduction of 14.7% from baseline [χ²(9)=14.8, P=0.09]. The decrease in uptake was noticeable in all ROIs during the washout phase of the blocking scans, from 20-100.5 min after the administration of [18F]PK-209. The regional rank order and peak values of radioactivity concentration were unaltered by the administration of MK-801 [χ²(9)=16.6, P>0.05]. Peak uptake values, however, were observed earlier than baseline during the blocking scans [χ²(9)=19.6, P<0.05; Friedman test]. For the whole-brain, radioactivity uptake under blocking conditions peaked at 8.8±2.6 min, and was equivalent to 0.029±0.001 %ID/cm³.

### 5.3.6. Effect of MK-801 administration on the VT of [18F]PK-209

Table 2 shows individual VT values of [18F]PK-209 for all ROIs examined, at baseline and after MK-801 administration. Parametric images were obtained by plasma-input Logan analysis and are shown for subject 1 in Figure 5a. The rank order of regional VT values (mL/cm³) at baseline was striatum (12.9±1.2) and cerebellum (11.5±1.0) > temporal (10.9±0.4), occipital (10.9±1.2), and frontal lobes (10.5±0.8). Between-subject variability in quantifying the VT of [18F]PK-209 ranged from 19.0% in the occipital, to 8.9% in the frontal lobe.

Table 2. VT of [18F]PK-209, at baseline and after the administration of MK-801 (0.3 mg·kg⁻¹).

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline VT</td>
<td>Blocked VT</td>
<td>Baseline VT</td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>5.1 ± 0.4</td>
<td>10.8</td>
<td>8.9</td>
</tr>
<tr>
<td>Occipital lobe</td>
<td>7.2 ± 0.8</td>
<td>8.8</td>
<td>7.9</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>3.7 ± 0.3</td>
<td>10.2</td>
<td>9.0</td>
</tr>
<tr>
<td>Striatum (caudate &amp; putamen)</td>
<td>1.3 ± 0.1</td>
<td>11.3</td>
<td>9.3</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>4.0 ± 0.1</td>
<td>10.4</td>
<td>7.7</td>
</tr>
<tr>
<td>Whole brain</td>
<td>97.0 ± 2.5</td>
<td>10.1</td>
<td>9.6</td>
</tr>
<tr>
<td>Mean VT in frontal,</td>
<td>10.3 ± 0.4</td>
<td>8.6 ± 0.3*</td>
<td>11.3 ± 0.3</td>
</tr>
<tr>
<td>occipital, temporal lobes,</td>
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<tr>
<td>the striatum and the</td>
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<td>cerebellum</td>
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* Individual values from left and right ROIs were derived separately, and averaged within each subject before calculating mean ± SEM values of VT in each brain region.

* P < 0.05 vs. baseline condition, Wilcoxon matched-pair tests.
Mean \( V_T \) values in all brain areas analysed decreased from 11.3±0.4 mL/cm\(^3\) at baseline to 9.7±0.8 mL/cm\(^3\) after MK-801 administration [\( \chi^2(9) = 11.4, P > 0.05; \) Friedman test]. The effect of pretreatment with MK-801 on the \( V_T \) of \([^{18}F]PK-209 \) varied between subjects. Brain regional \( V_T \) values were overall reduced compared with baseline for subjects 1 and 2 (Z=2.0, \( P < 0.05 \)), and increased for subject 3 (Z=2.0, \( P < 0.05 \)). In all cases, Logan plots became linear from 10 min post injection of \([^{18}F]PK-209 \) (\( t^* = 10 \) min; Figure 5b).

Figure 5. Total distribution volume (\( V_T \)) images (A) and whole-brain Logan plots (B) of \([^{18}F]PK-209 \) for subject 1, at baseline and after the administration of MK-801. Dynamic PET scans from propofol-anaesthetised monkeys were acquired over a period of 120 min. Parametric images were generated using plasma-input Logan graphical analysis, and are presented through the transaxial plane from areas of the cortex to the cerebellum. Images have been smoothed by isotropic Gaussian filter of 2 mm at full width at half maximum. Brain regional \( V_T \) values were overall reduced compared with baseline for subjects 1 and 2, and increased for subject 3.
5.4. Discussion

The present study was designed to evaluate whether $[^{18}F]PK-209$ is a suitable tracer for imaging the ion-channel site of NMDAr's. We show that PK-209 targets the intrachannel site with high apparent affinity and selectivity, and provide evidence that PET-measurable quantification of the NMDAr is feasible in the rhesus monkey brain using $[^{18}F]PK-209$.

Only a limited number of ion-channel radiotracers have been tested for their ability to visualize NMDAr's in the nonhuman primate brain [9-10]. These include radiolabeled analogues of MK-801, tenocyclidine (TCP), ketamine, memantine, and the guanidine GMOM, a ligand that has been labeled with carbon-11 and is structurally closely related with PK-209 [20]. The radiochemical synthesis advantages of $[^{18}F]PK-209$ over $[^{11}C]GMOM$ range from suitability of labeling PK-209 with fluorine-18, to rapidly delivering $[^{18}F]PK-209$ in adequate radiochemical yields (>20%, decay-corrected) and with high specific activity ($≈100$ GBq/µmol). From a pharmacological perspective, the minimal off-target effects observed on a broad selectivity screen render PK-209 one of the most selective ligands available to date for the ion-channel site of NMDAr's. The compound’s selectivity profile is comparable with that of the reference standard for non-competitive NMDAr antagonism, MK-801 [21]. In addition, the commercially obtained apparent affinity value for PK-209 ($K_i=22$ nM) is in the range previously reported by our group ($K_i=18$ nM), confirming the ligand's high affinity for the intrachannel site of NMDAr's [12]. Taken together, these in vitro results suggest that contrary to $^{18}$F-labeled derivatives of MK-801 [22], TCP [23] or memantine [24], the use of $[^{18}F]PK-209$ for PET imaging of the NMDAr is unlikely to be limited by poor tracer selectivity and/or potency.

The rate of $[^{18}F]PK-209$ metabolism was high in the monkey plasma, and similar to that reported for enantiomers of $[^{11}C]$ketamine [25] and $[^{11}C]$GMOM [20]. 10 min following $[^{18}F]PK-209$ administration, the percentage of plasma radioactivity attributable to unchanged tracer was already less than 50%, indicating that rapid metabolism hinders prolonged measurements of $[^{18}F]PK-209$ from arterial plasma. To reduce uncertainty in determining the plasma concentration of $[^{18}F]PK-209$, particularly during later time frames, the 1 minus polar radioactivity fraction was used as a surrogate of non-metabolised radiotracer. Two assumptions were accepted in taking this approach, namely that polar metabolites of $[^{18}F]PK-209$ do not cross the blood-brain barrier, and that the accumulation of lipophilic metabolites of $[^{18}F]PK-209$ in the monkey brain is low, if any at all. Using an identical method of analysis as in the present report [11], we have previously shown that polar metabolites of $[^{18}F]PK-209$ hardly enter the mouse brain ($≈4\%$ contribution to total brain radioactivity, 60 min
after radioligand injection). Since over the 2 h course of these scans 75.0% and 19.2% of plasma radioactivity was associated with polar metabolites and parent tracer, respectively, a potential contribution of lipophilic metabolites to the PET signal of $^{[18F]}$PK-209 is indeed expected to be low. The reversible apparent kinetics in the TACs, and the fact that linearity was attained from early to late time-points in the Logan plots, further argue against the significant accumulation of $^{[18F]}$PK-209-derived metabolites in the rhesus brain.

$^{[18F]}$PK-209 entered the brain readily, showing a fairly uniform distribution in the brain areas examined, namely between the cortex, striatum and the cerebellum. Due to the low signal-to-noise ratio in the dynamic PET images, and in order to perform a user-independent analysis, small ROIs were not delineated in the current study. Instead, the uptake of $^{[18F]}$PK-209 was quantified in large brain areas, which are known to exhibit high (cortex), moderate (striatum) and low (cerebellum) levels of NMDAr expression, in vitro [26]. The regional variation of $V_T$ estimates at baseline was <20%, indicating acceptable between-subject variability in quantifying the uptake of $^{[18F]}$PK-209 using Logan plots. In addition, prolonged retention of radioactivity during the baseline scans was clearly observed in NMDAr-rich regions relative to the cerebellum, suggesting specific receptor targeting. However, the rank order of regional $^{[18F]}$PK-209 distribution was at odds with the rank order of NMDAr density predicted from in vitro studies, since higher $V_T$ values were observed in the striatum and the cerebellum rather than the cortex.

The discrepancy between the in vitro and PET-measurable distribution of NMDArs has been repeatedly noted in the literature [22,24,27,28]. Perhaps the most convincing explanation hypothesized to account for the discrepant observations relates to the state of NMDAr activation. The in vitro preparations used to visualize the localisation of NMDArs are likely to contain concentrations of endogenous agonists that are high enough to fully activate the receptors. This is evident from autoradiographic experiments, in which exogenously applied glutamate and glycine do not enhance the binding of $[^3H]$MK-801 to NMDArs above baseline levels [29-30]. Since access to the ion-channel site depends on the concentration of glutamate and glycine [7], the in vitro binding of radioligands to maximally activated receptors may not necessarily reflect their in vivo uptake, and thus correspond to the PET-measurable distribution of NMDArs. In addition, limited access to the intrachannel site in the living brain can be anticipated under physiological conditions, since NMDAr overactivation has been associated with several pathological processes. Further explanations for the discrepancies in NMDAr distribution between in vitro and imaging studies may include differences in ion-channel opening times between brain regions [31], which can only influence the uptake of radioligands in vivo, the presence
of two binding sites within the ion-channel of NMDArs [32], which might be differentially available between in vitro and in vivo conditions [33], and less likely - differences in regional NMDAr expression between rodent and primate species. In any case, the pattern of NMDAr distribution in the rhesus brain is consistent with that observed in humans, using ion-channel tracers such as [123I]CNS1261 [34] or [18F]GE-179 [28]. Hence, our results confirm the discrepancies between the in vitro and imaging experiments, and invite further consideration of the mechanisms that may give rise to them.

Pretreatment with MK-801 30 min before the administration of [18F]PK-209 shifted the peak of the time-activity curves to the left compared with baseline, without altering the maximum radioactivity concentration values. The increased uptake of radioactivity during the initial time frames of the blocking scans could be related to MK-801-induced increases in regional cerebral blood flow [35], suggesting that the distribution of [18F]PK-209 may be partially perfusion-dependent. However, since there was no change in the peak amplitude of radioactivity concentrations compared with baseline, a contribution of tracer efflux to the shape of the time-activity curves, alone or in combination with other kinetic parameters, cannot be excluded. While these observations require further investigation, the Logan plot analysis we employed provides an estimate of $V_T$ at equilibrium that is perfusion-independent, suggesting that possible MK-801-induced alterations in cerebral blood flow are unlikely to have influenced the $V_T$ values of [18F]PK-209 under blocking conditions. In support of this suggestion, leftward shifts in uptake were consistently observed during the blocking scans, while the effect of pretreatment with MK-801 on the $V_T$ of [18F]PK-209 varied between subjects.

Logan-derived $V_T$ was overall decreased compared with baseline for subjects 1 and 2, and increased for subject 3. It is unlikely that these changes are secondary to MK-801-induced alterations in the peripheral pharmacokinetics of [18F]PK-209, since the profile of metabolite formation, the distribution between plasma and whole-blood, the arterial plasma concentration, and the clearance of [18F]PK-209 at baseline were unaffected by the administration of MK-801. Moreover, while binding to plasma proteins was not measured in this study, it is unclear how pretreatment with MK-801 may both have increased and decreased the plasma-free concentration of [18F]PK-209, leading to inconsistent alterations in its $V_T$ compared with baseline. Therefore, the effects of MK-801 administration on the uptake of [18F]PK-209 are probably due to pharmacodynamic, rather than pharmacokinetic reasons. To the best of our knowledge, this is the first nonhuman primate study to demonstrate decreases in the $V_T$ of an intrachannel PET tracer following the administration of the highly selective ion-channel blocker MK-801, suggesting that [18F]PK-209 can visualize and quantify
the NMDAr in vivo. Nevertheless, a note of caution should be inserted in interpreting the results of the blocking scans, since the reason for increased $V_T$ values in subject 3 is unknown.

One possible explanation may involve alterations in the target of interest itself, i.e. changes in the functional state of NMDArs, which may have occurred in the interval between the baseline and blocking scans of subject 3. Following its baseline scan, subject 3 was administered for 3 days with the partial $\mu$-opioid receptor (MOP) agonist buprenorphine, for welfare reasons (0.02 mg/kg, twice daily). Agonists and partial agonists at the MOP, however, are known to increase the function of NMDArs [36], in brain regions where the two receptors colocalise and may physically interact, including the striatum, cortex, and - to a smaller extent - the cerebellum [37]. Moreover, it is relatively well-established that the potentiating effects of MOP agonism on the function of NMDArs persist long after the end of opioid administration, both at the neurochemical and behavioural levels. For example, the binding of [$^3$H]MK-801 is increased in the brain of rats that have been withdrawn from treatment with partial MOP agonists [38], while the persistent functional potentiation of NMDArs plays a central role in opioid-induced analgesic tolerance and physical dependence [39]. Therefore, the increase in the $V_T$ of $[^{18}\text{F}]PK-209$ in the buprenorphine-treated subject may be associated with opioid-induced increases in NMDAr function. In support of this suggestion, confounding effects of MOP agonism on blocking the uptake of ‘state-dependent’ NMDAr tracers have been documented previously in fentanyl-anaesthetized mice [40]. In addition, the relatively low blocking dose of MK-801 used in the present study is unlikely to have produced free brain concentrations of the drug that are high enough to occupy the entire population of NMDArs, thereby compensating for any potential increases in the receptor’s function after the administration of buprenorphine [41]. Thus, it is intriguing to speculate that the increased uptake of $[^{18}\text{F}]PK-209$ compared with baseline in subject 3 may actually reflect the radioligand’s ability to quantify alterations in the functional state of NMDArs.
5.5. Conclusions

PK-209 binds to the ion-channel site of NMDArs with high apparent affinity and selectivity. Although the rate of metabolism is high, the regional $V_T$ of $[^{18}F]PK-209$ can be quantified in the primate brain with acceptable between-subject variability. Evidence for specific targeting of the NMDArs in vivo is modest but present in the current study, both at baseline and after the administration of MK-801. Further evaluation of $[^{18}F]PK-209$ in humans is warranted.

5.6. Acknowledgements

This work was supported by the Center for Translational Molecular Medicine (http://www.ctmm.nl), project LeARN (02N-101). Part of the work was also supported by the European Union’s Seventh Framework Programme (FP7/2007-2013), grant agreement n° HEALTH-F2-2011-278850 (INMiND).

We thank Rolph van Kooij for his technical assistance in preparing $[^{18}F]PK-209$ and the staff of BV Cyclotron VU, Amsterdam, for supplying $^{18}F$. We are grateful to Inge de Greeuw and Mariska Verlaan for their assistance in operating the HRRT scanner.

5.7. Supplementary data

Figure S1. Summed images of radioactivity uptake, from 0-120 min after the administration of $[^{18}F]PK-209$ to subject 2. No radioactivity accumulation was observed in bone-containing structures, indicating the minimal defluorination metabolism of $[^{18}F]PK-209$. 
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<tr>
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10 μM of PK-209 were tested against all of the above targets, in competition binding assays against agonist or antagonist radioligands.

(h): Recombinant or endogenously expressed human receptors.
5.8. References


Synthesis and preclinical evaluation of carbon-11 labelled N-((5-(4-fluoro-2-[\textsuperscript{11}C]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine as a PET tracer for NR2B subunit-containing NMDA receptors

Johannes AM Christiaans, Pieter J Klein, Athanasios Metaxas, Esther JM Kooijman, Robert C Schuit, Josée E Leysen, Adriaan A Lammertsma, Bart NM van Berckel, Albert D Windhorst

Published in: *Nuclear Medicine and Biology* 2014;41:670-680
Abstract

*Introduction:* The N-methyl-D-Aspartate (NMDA) receptor plays an important role in learning and memory. Overactivation is thought to play an important role in neurodegenerative disorders such as Alzheimer’s disease. Currently, it is not possible to assess *N*-methyl-D-aspartate receptor (NMDAr) bio-availability *in vivo*. The purpose of this study was to develop a positron emission tomography (PET) ligand for the NR2B binding site of the NMDA receptor.

*Methods:* N-((5-(4-fluoro-2-methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine was radiolabelled with carbon-11 in the phenyl moiety. Biodistribution and blocking studies were carried out in anaesthetized mice and in non-anaesthetized rats.

*Results:* N-((5-(4-fluoro-2-[11C]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine was prepared in 49 ± 3% (decay-corrected) yield, affording 4.1 ± 0.3 GBq of formulated product at the end of synthesis with a radiochemical purity of >99% and with a specific activity of 78 ± 10 GBq/µmol.

*Conclusion:* A new NR2B PET ligand was developed in high yield. [11C]82 readily enters the brain and binds to the NR2B subunit-containing NMDAr in the rodent brain. High sigma-1 receptor binding may, however, limit its future application as a PET probe for imaging the NR2B subunit-containing NMDAr. Anesthesia has an effect on NMDAr function and therefore can complicate interpretation of preclinical *in vivo* results. In addition, effects of endogenous compounds cannot be excluded. Despite these potential limitations, further studies are warranted to investigate the value of [11C]82 as an NR2B PET ligand.
6.1. Introduction

The N-methyl-D-aspartate receptor (NMDAr) belongs to the class of ionotropic glutamate receptors (iGluRs). These iGluRs are named after their selective agonists, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid and N-methyl-D-aspartate (NMDA). The structure and function of the NMDAr have been reviewed extensively over the years [1-3]. NMDAr dysfunction is present in a wide range of neurological and psychiatric diseases [4-7]. In neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease and schizophrenia, NMDAr overexpression may be present, which in turn could lead to excitotoxicity [8,9]. In Alzheimer’s disease, NMDAr dysfunction can also occur due to amyloid-β peptide (Aβ) oligomers [10].

In the last decade, a major effort in NMDAr drug development has focussed on antagonists for the N-methyl-D-aspartate receptor subtype 2B (NR2B) binding site as possible therapeutics in a wide range of CNS pathologies, including acute and chronic pain, stroke, head trauma, drug induced dyskinesias, and dementias [11]. It has been speculated that selective antagonists of the NR2B subtype might provide a cleaner side effect profile compared with antagonists of the glycine binding site or blockers of the ion channel [12].

In vivo quantification of the NMDAr using positron emission tomography (PET) would be a useful tool for assessing both NMDAr availability and its aetiology in these neurodegenerative diseases. Furthermore, in vivo imaging of the NMDAr could be an instrument for image guided pharmacotherapy. Recently, the development of PET and SPECT tracers for imaging the NMDAr system has been reviewed [13]. Unfortunately, the majority of radiotracers listed exhibited poor in vivo applicability. Common problems were poor brain penetration, non-specific uptake in the brain and extensive metabolism [14,15,16].

To develop a radiotracer for the NMDAr, that can successfully be applied in vivo in man, several factors come into play. First, there are the usual requirements for radiosynthesis and the radioligand itself, which needs to have suitable properties for uptake in the brain, receptor binding kinetics and metabolic stability. Second, there is the complex system of NMDAr activation, for which several endogenous ligands (e.g. glutamate, glycine, serine, agents acting at the spermidine site) play a role in regulating channel opening. Exogenous ligands can bind to several sites at the NMDAr and their binding may be dependent on the activation of the receptor by endogenous ligands. Finally, in vivo testing of radiotracers in laboratory animals requires anaesthesia, which might interfere with NMDAr functioning.
At present, there is no well validated NMDAr NR2B PET radioligand available. Recently, a new class of NR2B antagonists with a wide range of potential 2,6-disubstituted aromatic and heteroaromatic compounds was described, which may be useful for treating depression [17]. This class of NR2B antagonists offers a good opportunity to develop a radiotracer for investigating the NMDAr complex.

The aim of the present study was to synthesize N-((5-(4-fluoro-2-[11C]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine and evaluate its potential as an NMDAr-NR2B selective PET ligand.

6.2. Materials and methods

6.2.1. Materials

Chemicals were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands) or Acros Organics (Geel, Belgium) and were used without further purification. Ro25-6981 maleate was purchased from TEBU-Bio (Heerhuogwaard, the Netherlands). Ifenprodil tartrate and GBR12909 dihydrochloride were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). [3H]Ifenprodil was purchased from Perkin Elmer (Groningen, the Netherlands). High-performance liquid chromatography (HPLC) solvents (HPLC grade) were purchased from J.T. Baker (Valkenswaard, the Netherlands).

All reactions were carried out under argon atmosphere, unless stated otherwise. [11C]CO₂ was produced using an IBA Cyclone 18/9 cyclotron (Louvain-La-Neuve, Belgium) and [11C]methyl iodide was produced as described previously [18]. Radiosyntheses were performed using home-made synthesis modules [19].

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker AC 250 or 500 (Billerica, USA). Chemical shifts (δ) were defined relative to the signal of the solvent (7.27 for CDCl₃, 2.50 for DMSO-d₆). High Resolution Mass Spectrometry (HRMS) was performed using a Bruker MicroTOFQ with ESI (electrospray ionization) in a positive mode (Billerica, USA). Samples were injected (10 µL) in a liquid flow of methanol/water 1/1) at a flow of 100 µL-min⁻¹.

The preparative high-performance liquid chromatography (HPLC) system consisted of a Jasco PU-2089 HPLC pump (Jasco Benelux, de Meern, the Netherlands), a Rheodyne injector with a 20 µl loop (Thermo Fischer Scientific, Breda, the Netherlands), a Jasco UV-2075 Plus UV detector set at a wavelength of 254 nm (Jasco Benelux, de Meern, the Netherlands) and a Raytest Na(I) radioactivity detector (Raytest, Straubenhardt, Germany). The HPLC data were acquired and integrated using the software package ChromNAV 1.14 (Jasco Benelux, de Meern, the Netherlands).
Synthesis and preclinical evaluation of carbon-11 labelled N-((5-(4-fluoro-2-[11C]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine as a PET tracer for NR2B subunit-containing NMDA receptors

Netherlands. A Reprosphere 100 C18-DE 5 µm, 50 x 8 mm column (Dr Maisch GmbH, Ammerbuch-Entringen, Germany) at a wavelength of 254 nm and an eluent of acetonitrile/water/N,N-diisopropylethylamine 40/60/0.05 (v/v/v) was used with a flow of 3 mL·min⁻¹ (method A).

The analytical HPLC system was the same as the preparative HPLC system. The HPLC data were acquired and integrated using the software package GINA 5.01 (Raytest, Straubenhardt, Germany). A GraceSmart RP18 5u, 250 x 4.6 mm (Grace Alltech, Breda, the Netherlands) column at a wavelength of 254 nm with an eluent containing acetonitrile/water/trifluoroacetic acid 15/85/0.15 (v/v/v) and a flow of 1 mL·min⁻¹ was used (method B).

For metabolite analysis, a Phenomenex Gemini C18, 10*250 mm, 5 µm (Phenomenex, Utrecht, the Netherlands) column was used. The mobile phase was A = acetonitrile B = H₂O / 0,1 % ammonium acetate in a gradient system from 70% B to 10% B in 9 minutes at a flow of 3 mL·min⁻¹ (method C). The HPLC system consisted of Dionex Ultimate 3000 HPLC system (Dionex, Breda, the Netherlands), equipped with a Rheodyne injector with a 1 mL loop (Thermo Fischer Scientific, Breda, the Netherlands) and a Raytest Na(I) radioactivity detector (Raytest, Straubenhardt, Germany). The HPLC system was controlled using the software package Chromeleon 6.80 (Dionex, Breda, the Netherlands). Fractions of 30 s were collected and counted using a Perking Elmer 2480 Singlewell gammacounter (Groningen, the Netherlands). Chromatograms were generated using Microsoft Excel version 2007.

For in vivo studies, Wistar rats were obtained from Harlan (the Netherlands) and housed in groups of four to six per cage until treatment. B6C3F1/J mice were obtained from the Jackson Laboratory (USA) and were housed in groups of four to six per cage until treatment. All animals were kept at a constant temperature of 21 °C and under a 12 h light/dark cycle, in which lights were switched on at 8:00 a.m. Animals had unrestricted access to food (Teklad Global 16% Protein Rodent Diet, Harlan, Madison, WI, USA) and water. All animal experiments were performed in compliance with Dutch laws on animal experimentation and after approval by the local animal ethics committee.

The pharmacological selectivity was evaluated against a panel of targets in competition binding assays against agonist or antagonist radioligands (Cerep, Poitier, France).
6.2.2. Synthesis

6.2.2.1. 5-(4-fluoro-2-methoxyphenyl)nicotinaldehyde (81)

5-bromonicotinaldehyde 79 (897 mg, 4.83 mmol) was dissolved in 1,2-dimethoxyethane/ethanol/H_2O (7/2/1 v/v/v, 5 mL) and treated with 4-fluoro-2-methoxyphenylboronic acid 80 (820 mg, 4.83 mmol) followed by cesium carbonate (1.71 g, 5.26 mmol) and PdCl_2(dppf)-CH_2Cl_2 complex (141 mg, 0.193 mmol). The reaction mixture was heated at 80 °C for 12 h, and subsequently partitioned between ethyl acetate and H_2O. The organic layer was washed with saturated NaHCO_3, saturated NaCl, dried over anhydrous MgSO_4, filtered and concentrated under reduced pressure to give a dark brown oil (938 mg). This oil was purified by flash chromatography using a gradient of 0-40% ethyl acetate in n-hexanes. 5-(4-fluoro-2-methoxyphenyl)nicotinaldehyde 81 was obtained as a white solid (657 mg, 2.84 mmol, 58.9% yield). TLC in n-hexane/ethyl acetate 60/40 v/v; R_f = 0.37 product. ^1H-NMR (CDCl_3): δ = 3.83 (s, 3H, CH_3), 6.73-6.82 (m, 2H, 2 x Φ-H), 7.27-7.33 (m, 1H, Φ-H), 8.27 (s, 1H, Φ-H), 8.94 (s, 1H, Φ-H), 9.00 (s, 1H, Φ-H), 10.17 (s, 1H, aldehyde-H).

6.2.2.2. N-((5-(4-fluoro-2-methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine dihydro chloride (82)

5-(4-fluoro-2-methoxyphenyl)nicotinaldehyde 81 (300 mg, 1.30 mmol) was dissolved in 3 mL of dichloromethane along with cyclopentanamine (0.192 mL, 1.95 mmol). The solution was cooled in an ice bath, and sodium triacetoxyborohydride (319 mg, 1.51 mmol) was added. The reaction was stirred at room temperature for 16 h, and subsequently diluted with dichloromethane (50 mL). The resultant mixture was then washed with saturated NaHCO_3 (20 mL) and brine (20 mL). The combined organic fractions were concentrated and purified by flash column chromatography using a gradient of 0-5% of methanol in dichloromethane. N-((5-(4-fluoro-2-methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine 82 was obtained as a yellow oil (187 mg, 48.0%). This oil was dissolved in diethyl ether (20 mL) and 3 mL 1M HCl in diethyl ether was added drop-wise. The suspension was stirred for 30 minutes. The solid was filtered and washed with diethyl ether (50 mL) and dried under vacuum to give N-((5-(4-fluoro-2-methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine dihydrochloride as a white solid (196 mg, 0.525 mmol, 40.5% yield). ^1H-NMR of free base in CDCl_3: δ = 1.28-1.46 (m, 2H, CH_2 of c-pentyl), 1.48-1.51 (m, 2H, CH_2 of c-pentyl), 1.61-1.67 (m, 2H, CH_2 of c-pentyl), 1.76-1.83 (m, 2H, CH_2 of c-pentyl), 1.99 (bs, 1H, NH), 3.03-3.13 (m, 1H, CH of c-pentyl), 3.72 (s, 3H, CH_3), 3.76 (s, 2H, CH_2-N), 6.63-6.71 (m, 2H, 2 x Φ-H), 7.16-7.26 (s, 1H, Φ-H), 7.72 (s, 1H, Φ-H), 8.43 (s, 1H, Φ-H), 8.53 (s, 1H, Φ-H). ^1H-NMR (2HCl salt in DMSO-d_6) [500 MHz]: δ = 1.51-1.55 (m, 2H, CH_2 of c-pentyl), 1.72-1.84 (m, 4H, 2 x CH_2 of c-pentyl), 1.98-2.03 (m, 2H, CH_2 of c-pentyl),
3.51-3.54 (m, 1H, CH of c-pentyl), 3.86 (s, 3H, CH3), 4.35 (t, 2H, CH2-N, J1,2=5.97 Hz), 6.98-7.02 (dt, 1H, Φ-H, J1,2 = 2.36 Hz, JH-F = 11.34 Hz), 7.15-7.18 (dd, 1H, Φ-H, J1,2 = 2.40 Hz, JH-F = 8.40 Hz), 7.63-7.66 (m, 1H, Φ-H), 8.75 (s, 1H, pyridyl-H), 8.98 (s, 2H, 2 x pyridyl-H), 9.89 (s, 2H, NHz+). 13C-NMR (2HCl salt in DMSO-d6) [500 MHz]: δ = 23.72 (2 x CH2 of c-pentyl), 29.07 (2 x CH2 of c-pentyl), 45.72 (CH2-N), 56.45 (CH3), 58.54 (CH of c-pentyl), 100.46 (CH-Φ, J=26.2 Hz), 107.75 (CH-Φ, J=21.7 Hz), 120.05 (Φ-C, J=3.0 Hz), 130.41 (C-Φ), 132.08 (CH-Φ, J=10.3 Hz), 134.35 (Φ-C), 143.99 (CH-pyridyl), 144.43 (CH-pyridyl), 154.53 (CH-pyridyl), 157.71 (C-Φ, J=10.6 Hz), 163.83 (C-F, Jc,f=246.7 Hz). HR-MS: [M+H]+ = 301.1702 found; [M+H]+ = 301.1711 calc.

6.2.2.3. 2-(5-((cyclopentylamino)methyl)pyridin-3-yl)-5-fluorophenol (83)

To a solution of N-((5-(4-fluoro-2-methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine 82 (420 mg, 1.40 mmol) in 10 mL of dry dichloromethane was added boron tribromide in dichloromethane (1M) (6.99 mL, 6.99 mmol) drop-wise at -78 °C. After addition, the yellow reaction mixture was stirred for 16 h. The reaction was quenched by addition of 5 mL of methanol, 20 mL of water and 100 mL of ethyl acetate at 0 °C. The mixture was partitioned and the aqueous layer extracted with ethyl acetate (2 x 20 mL). The combined extracts were dried with anhydrous MgSO4 and evaporated to dryness. The solid was suspended in dichloromethane, filtered and washed with DCM and dried under vacuum at 40 °C to give 2-(5-((cyclopentylamino)methyl)pyridin-3-yl)-5-fluorophenol 83 as a white solid (298 mg, 1.04 mmol, 74.4 % yield). 1H-NMR in DMSO-d6 [500 MHz]: δ = 1.56-1.77 (m, 6H, 3 x CH2 of c-pentyl), 2.00-2.05 (m, 2H, CH2 of c-pentyl), 3.60-3.62 (m, 1H, CH of c-pentyl), 4.33-4.46 (m, 2H, CH2-N), 6.87-6.91 (m, 2H, 2 x Φ-H), 7.60-7.63 (m, H, Φ-H), 8.83-9.13 (m, 4H, 1 x NH + 3 x Φ-H), 9.17-9.25 (m, H, Φ-H), 10.88 (m, 1H, OH). 13C-NMR (2HCl salt in DMSO-d6) [500 MHz]: δ = 23.79 (2 x CH2 of c-pentyl), 29.21 (2 x CH2 of c-pentyl), 45.77 (CH2-N), 58.82 (CH of c-pentyl), 103.30 (CH-Φ, J=24.1 Hz), 107.06 (CH-Φ, J=21.8 Hz), 117.91 (Φ-C, J=2.4 Hz), 130.71 (C-Φ), 132.12 (CH-Φ, J=10.6 Hz), 135.28 (Φ-C), 142.61 (CH-pyridyl), 143.48 (CH-pyridyl), 156.32 (C-Φ, J=11.6 Hz), 163.465 (C-F, Jc,f=246.9 Hz). HR-MS: [M+H]+ = 287.1549 found; [M+H]+ = 287.1554 calc.
6.2.3. Radiosynthesis of N-((5-(4-fluoro-2-[11C]methoxyphenyl)pyridin-3-yl)methyl)cyclopentan-amine ([11C]82)

The automated synthesis was performed by trapping of [11C]methyliodide into a solution of 2-(5-((cyclopentylamino)methyl)pyridin-3-yl)-5-fluorophenol 83 (1 mg, 3.5 µmol) in dimethylsulfoxide (300 µL) in the presence of potassium carbonate (10 mg, 72 µmol) for 5 minutes at 85 °C. After reaction, the mixture was quenched with 40% acetonitrile in water containing 0.05% N,N-diisopropylethylamine (500 µL) before purification by preparative HPLC. The fraction containing the product was collected and diluted with water (40 mL). This solution was concentrated on a tC-18 plus Sep-pak (Waters Corporation, Milford MA, USA), rinsed with water (20 mL), subsequently eluted with sterile ethanol (96%, 1 mL) and diluted with a sterile and pyrogen free solution of 7.11 mM NaH2PO4 in 0.9% NaCl (w/v in water), pH 5.2 (9 mL). Radiochemical purity of the product was determined by HPLC (method B). The retention time of N-((5-(4-fluoro-2-[11C]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine [11C]82 was 8.64 min, which was confirmed using N-((5-(4-fluoro-2-methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine 82 as reference.

6.2.4. Determination of $\text{LogD}_{\text{oct},7.4}$

The distribution of the radio labelled compound between 1-octanol and 0.2 M phosphate buffer (pH = 7.4) was measured in triplicate at room temperature. Briefly, 1 mL of a 20 MBq/mL solution of the radiolabeled compound in 0.2 M phosphate buffer (pH = 7.4) was vigorously mixed with 1 mL of 1-octanol for 1 min at room temperature using a vortex. After a settling period of 30 min, five samples of 100 µL were taken from both layers. For determining recovery, 5 samples of 100 µL were taken from the 20 MBq/mL solution. All samples were counted for radioactivity. The $\text{LogD}_{\text{oct},7.4}$ value was calculated according to $\text{LogD}_{\text{oct},7.4} = 10\log(A_{\text{oct}} / A_{\text{buffer}})$, where $A_{\text{oct}}$ and $A_{\text{buffer}}$ represent average radioactivity counts of the five 1-octanol and buffer samples.

6.2.5. Membrane Preparation

Male Wistar rats (180-200 g) were killed by decapitation. Forebrains were removed rapidly and homogenized using a DUALL tissue homogenizer (10 strokes, 2000 rpm) (VWR, Amsterdam, the Netherlands), in a 10-fold excess (v/w) of an ice cold 0.25 M sucrose solution. Nuclei and cell debris were removed by centrifugation (10 min x 400 x g) in a Beckman-Coulter refrigerated ultracentrifuge (rotor 60Ti) (Woerden, the Netherlands). The resulting pellet was rehomogenized in 5 vol 0.25 M sucrose solution.
solution and re-centrifuged under the same conditions. The combined supernatants were diluted in Tris-acetate buffer (50 mM, pH 7.4) to a final dilution of 40 v/w and centrifuged for 30 min x 200,000 x g, in order to obtain membranes from the cell surface, mitochondrial, and microsomal fractions. The pellet was re-suspended in 20 volumes of Tris-HCl + 0.01 % Triton buffer (pH 7.4), kept at 37 °C for 10 min, and centrifuged under the same conditions. The final pellet was suspended in Tris-HCl buffer (dilution 5, pH 7.4) and stored at -80 °C in 5 mL aliquots. On the day of each experiment, membranes were thawed to room temperature and washed twice by centrifugation (30 min x 200,000 x g). After the final centrifugation, pellets were suspended in 80 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.4).

6.2.6. Competition binding assay

Affinities of N-((5-(4-fluoro-2-methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine 82, precursor compound 83 and reference compound Ro25-6981 were determined by measuring the ability of various concentrations of unlabelled ligand to inhibit specific binding of 5 nM [3H]ifenprodil (specific activity: 40 Ci/mmol; 1480GBq/mmol). Unlabelled ligands were dissolved as 10 mM stock solutions in DMSO and used in a concentration range from 10^-4 to 10^-12 M. Competition binding experiments were carried out at 4°C, in a final assay volume of 500 µL assay buffer (50 mM Tris-HCl, Ph 7.4), containing 5 µM GBR12909 to block σ receptor binding. The incubation mixture was composed of 400 µL of the membrane suspension, containing a total amount of 2.5 mg original wet weight of tissue, 50 µL [3H]ifenprodil, 45 µL assay buffer (50 mM Tris-HCl, pH 7.4) and 5 µL of unlabelled drug solution (final DMSO concentration: 1%). Non-specific binding was determined in the presence of 10 µM Ro25-6981. Incubations were terminated after 2 h by filtration through 0.3 % PEI-embedded Whatman GF/B filters (‘s-Hertogenbosch, the Netherlands), using a 48-well Brandel harvester (Alpha Biotech, Glasgow, UK). Filters were washed three times with 3 mL of ice cold Tris-HCl buffer (pH 7.4), and radioactivity retained on the filter was determined by liquid scintillation spectrometry in 5 mL of Optiphase-HiSafe 3 (Perkin Elmer, Groningen, the Netherlands), at an efficiency of 40%. K_i values were determined by nonlinear regression analysis using the equation: logEC_50 = log[10]logK_i*[1 + [3H]ifenprodil(nM)] / [3H]ifenprodil K_d(nM)], (GraphPad Software Inc, San Diego, CA). The concentration of [3H]ifenprodil was 5 nM and the applied K_d-value of 14.8 nM was obtained in radioligand saturation binding experiments (concentration range 1-400 nM), in assay conditions as described above.
6.2.7. *In vitro* autoradiography

Brain sections of drug-naïve, 7-9 weeks old, male B6C3F1/J mice were used for autoradiography of $[^{11}C]82$ binding sites ($n=3$). 20 µm thick coronal brain sections were thawed from -20 °C to room temperature, pre-washed in assay buffer (50 mM Tris-HCl, pH 7.4) for 2 x 10 min, and dried. Sections were then incubated at room temperature for 30 min, in assay buffer containing $[^{11}C]82$ (>30 nM; specific activity >30 GBq/µmol at $t_0$). To determine non-specific binding, a series of immediately adjacent brain sections was incubated in the same buffer, in the presence of 1 µM Ro25-6981. Incubations were terminated by 1 min washing into ice-cold assay buffer (pH 7.4), followed by a rapid rinse in ice cold demineralized water. Next, sections were air dried and opposed overnight to Kodak BioMax MR-1 film, which was developed in a Kodak D19 developer and fixed with Kodak rapid fixer (Sigma-Aldrich, Zwijndrecht, the Netherlands). Analysis of $[^{11}C]82$ binding was performed by video-based computerized densitometry, using an MCID image analyser (InterFocus Imaging, Linton, UK). Specific binding was quantified following subtraction of non-specific from total binding images. Values were calculated as the mean ± SEM relevant optical density (ROD) values of $[^{11}C]82$ binding.

6.2.8. Biodistribution of $[^{11}C]82$ in anaesthetized mice

Male, 7-9 weeks old B6C3F1/J mice were used for biodistribution experiments. Animals were anaesthetized with a 1:2:1 mixture of [fentanyl/fluanisone (HypnormTM; Vetapharma, Leeds, UK) and midazolam (DormicumTM; Actavis, Harnarfjordur, Iceland)] (ffm) in sterile water. The ffm mixture was administered intraperitoneally (11 mL/kg), 5 min prior to radiotracer administration. $[^{11}C]82$ (60.7 ± 3.4 MBq, specific activity 31.5 ± 8.3 GBq/µmol at $t_0$) was injected via the tail vein, in a saline solution containing 10% ethanol (5 mL/kg). Following radiotracer injection, mice were killed by cervical dislocation at 5, 15, 30 or 60 min ($n=4$/time point). At each time point, blood was obtained by heart puncture, and selected organs, including heart, liver, kidneys, lungs and brain, were removed. The brain was further dissected into frontal cortex, olfactory tubercle, striatum, cerebral cortex (bregma 1.70-0.02 mm), entorhinal cortex, hippocampus, hypothalamus, cerebellum, and pons/medulla oblongata. All organs and brain areas were weighed, and radioactivity was measured in a 1282 Compugamma CS (LKB Wallac, Turku, Finland), using 5 x 10 µL aliquots of the injected formulation as internal standard. Results are expressed as differential absorption ratio (DAR): (counts per minute (cpm)/g tissue)/(cpm injected/g body weight) corrected for decay to time of injection. Two-way repeated measures ANOVA (analysis of variance), followed by Fisher’s Least Significant Difference (LSD) post-hoc
comparisons, was used for analysis of radiotracer uptake in the various brain regions and organs at several time points.

6.2.9. Blocking of $[^{11}\text{C}]82$ in anaesthetized mice

To visualize distribution and selectivity of $[^{11}\text{C}]82$ for the NR2B binding site of NMDA receptors in vivo, B6C3F1/J mice were anaesthetized with ffm, as described in section 2.8. Pairs of mice were injected with either saline or Ro25-6981 (10.0 mg/kg, i.p.), 20 min before a tail-vein injection of $[^{11}\text{C}]82$ (70.1 ± 14.6 MBq, specific activity: 38.1 ± 7.9 GBq/μmol at $t_0$; n=4 pairs). 15 min after radiotracer injection, mice were killed by cervical dislocation and brains were removed, frozen in liquid nitrogen and processed for quantitative autoradiography. Coronal brain sections (20 μm) from control and Ro25-6981 treated mice were obtained at -21 °C in a Leica CM3050S cryostat (Leica Microsystems, Rijswijk, the Netherlands) and immediately opposed to phosphor storage screens. These screens were read in a Storm™ 860 Phosphorimager (GE Healthcare Europe GmbH, Diegem, Belgium) on the following day and images obtained were analyzed using ImageQuant TL 8.1 software (GE Healthcare Europe GmbH, Diegem, Belgium). A series of immediately adjacent brain sections were opposed to Kodak Biomax MR-1 film, which was developed after overnight exposure, to obtain high resolution images. Specific uptake was defined as the difference in brain accumulation of $[^{11}\text{C}]82$ between control and Ro25-6981 treated animals. Analysis of radiotracer uptake in control and blocked mice across different brain regions was performed using two-way ANOVA.

6.2.10. Metabolite analysis in anaesthetized mice

B6C3F1/J mice were anaesthetized with ffm as described in section 2.8. $[^{11}\text{C}]82$ (60.7 ± 3.4 MBq, specific activity 31.5 ± 8.3 GBq/μmol at $t_0$) was injected via the tail vein, in a saline solution containing 10% ethanol (5 mL/kg). About 0.6 mL of blood was collected via heart punctures in a heparin tube and centrifuged for 5 min at 4000 RPM (Hettich universal 16, Depex B.V, the Netherlands). Plasma was separated from blood cells, diluted 1:2 with demi water, and passed over an activated tC18 Sep-Pak cartridge (Waters, the Netherlands). The Sep-Pak cartridge (SPE) was washed with 5 mL of water. Recovered fractions were defined as the polar radiolabelled metabolite. Thereafter, the tC18 Sep-Pak cartridge was eluted with 1.5 mL of methanol followed by 1.5 mL of water. This eluate was defined as the non-polar fraction and was analysed using HPLC (method C) with online radioactivity detection.
6.2.11. Blocking of $[^{11}\text{C}]\text{82}$ in non-anaesthetized rats

Male Wistar rats (220-250 g) were injected with either saline or Ro25-6981 (10.0 mg/kg, i.p.), 30 min before a tail-vein injection of $[^{11}\text{C}]\text{82}$ (241.5 ± 120.1 MBq with a specific activity of 139.2 ± 0.3 GBq/µmol at $t_0$; n=2 pairs). 15 min after radioactivity injection, rats were killed by decapitation. Brains were removed and homogenized in 40 volumes (v/w) of 5 mM Tris-HCl buffer (pH 7.4), using a T18 Ultra Turrax homogenizer (Ika®, Staufen, Germany). 4x1 mL aliquots of the brain homogenate were filtered immediately through Whatman GF/B filters, presoaked in ice-cold buffer, using a 48-sample Brandel harvester (Alpha Biotech, Glasgow, UK). Filters were washed twice with 3 mL of ice cold 5 mM Tris-HCl buffer, and trapped radioactivity was quantified in a 1282 Compugamma CS counter, using 5 x 10 μL aliquots of the injected formulation as internal standard. Data are expressed as percentage injected dose/g original wet weight tissue (%ID/g) and were compared using two-tailed Student’s t-tests.

For autoradiography, pairs of male Wistar rats were injected with either saline or Ro25-6981 (20.0 mg/kg, i.p.), 30 min before a tail-vein injection of $[^{11}\text{C}]\text{82}$ (464.3 ± 167.5 MBq with a specific activity of 139.6 ± 0.3 GBq/µmol at $t_0$ (n=2 pairs). Rats were killed by decapitation 15 min after radioactivity injection, and their brains were removed and snap-frozen in liquid nitrogen for quantitative autoradiography. Coronal brain sections (20 µm) were obtained at -21 °C using a Leica CM3050S cryostat and they were immediately opposed to phosphor storage screens. On the following day, screens were read in a Storm™ 860 Phosphorimager and images obtained were analysed using ImageQuant TL 8.1 software. Data are expressed as mean ± SEM average pixel intensity of 2 rats/group and analysed using two-way ANOVA for the factors treatment and brain region.

6.2.12. Pharmacological screening

Binding selectivity assays were performed at Cerep (Cerep, Poitiers, France). A stock solution of 10 mM 82 in DMSO was send to Cerep and tested against an enzyme/receptor panel of 79 targets at a final concentration of 10 µM, according to the company’s materials and methods (Table 1).
Table 1. Enzyme/receptor panel of 79 targets used for pharmacological screening.

<table>
<thead>
<tr>
<th>Panel of Selectivity Assays Performed at Cerep</th>
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<tbody>
<tr>
<td>1 A1 (adenosine receptor) (h)</td>
<td>41 NK2 (tachykinin receptor) (h)</td>
</tr>
<tr>
<td>2 A2A (adenosine receptor) (h)</td>
<td>42 NK3 (tachykinin receptor) (h)</td>
</tr>
<tr>
<td>3 A3 (adenosine receptor) (h)</td>
<td>43 Y1 (neuropeptide Y1 receptor) (h)</td>
</tr>
<tr>
<td>4 alpha 1 (adrenergic receptor)</td>
<td>44 Y2 (neuropeptide Y2 receptor) (h)</td>
</tr>
<tr>
<td>5 alpha 1A (adrenergic receptor) (h)</td>
<td>45 NTS1 (neurotensin NT1 receptor) (h)</td>
</tr>
<tr>
<td>6 alpha 1B (adrenergic receptor) (h)</td>
<td>46 N neuronal alpha4beta2 (nicotinic receptor) (h)</td>
</tr>
<tr>
<td>7 alpha 2 (adrenergic receptor)</td>
<td>47 N neuronal alpha 7 (nicotinic receptor) (h)</td>
</tr>
<tr>
<td>8 alpha 2A (adrenergic receptor) (h)</td>
<td>48 delta 2 (DOP opioid receptor) (h)</td>
</tr>
<tr>
<td>9 alpha 2B (adrenergic receptor) (h)</td>
<td>49 kappa (KOP opioid receptor)</td>
</tr>
<tr>
<td>10 alpha 2C (adrenergic receptor) (h)</td>
<td>50 mu (MOP opioid receptor) (h)</td>
</tr>
<tr>
<td>11 beta 1 (adrenergic receptor) (h)</td>
<td>51 OX1 (orexin receptor) (h)</td>
</tr>
<tr>
<td>12 beta 2 (adrenergic receptor) (h)</td>
<td>52 OX2 (orexin receptor) (h)</td>
</tr>
<tr>
<td>13 AT1 (angiotensin receptor)</td>
<td>53 NOP (ORL1 nociceptin receptor) (h)</td>
</tr>
<tr>
<td>14 BZD (central; gabaergic receptor)</td>
<td>54 CXCR2 (interleukin-8B receptor) (h)</td>
</tr>
<tr>
<td>15 B2 (bradykinin receptor) (h)</td>
<td>55 EP4 (prostaglandin receptor) (h)</td>
</tr>
<tr>
<td>16 CB1 (cannabinoid receptor) (h)</td>
<td>56 P2X (purinergic receptor)</td>
</tr>
<tr>
<td>17 CCK1 (cholecystokinin receptor) (h)</td>
<td>57 P2Y (purinergic receptor)</td>
</tr>
<tr>
<td>18 D1 (dopaminergic receptor) (h)</td>
<td>58 5-HT1A (serotonergic receptor) (h)</td>
</tr>
<tr>
<td>19 D2S (dopaminergic receptor) (h)</td>
<td>59 5-HT1B (serotonergic receptor)</td>
</tr>
<tr>
<td>20 D2L (dopaminergic receptor) (h)</td>
<td>60 5-HT2A (serotonergic receptor) (h)</td>
</tr>
<tr>
<td>21 ETA (endothelin receptor) (h)</td>
<td>61 5-HT2B (serotonergic receptor) (h)</td>
</tr>
<tr>
<td>22 GABA</td>
<td>62 5-HT3 (serotonergic receptor) (h)</td>
</tr>
<tr>
<td>23 GAL2 (galanin receptor) (h)</td>
<td>63 5-HT5a (serotonergic receptor) (h)</td>
</tr>
<tr>
<td>24 GABAB (1b) (gabaergic receptor) (h)</td>
<td>64 5-HT6 (serotonergic receptor) (h)</td>
</tr>
<tr>
<td>25 AMPA receptor (glutamatergic receptor)</td>
<td>65 5-HT7 (serotonergic receptor) (h)</td>
</tr>
<tr>
<td>26 kainate receptor (glutamatergic receptor)</td>
<td>66 sigma receptor (h)</td>
</tr>
<tr>
<td>27 NMDA receptor (GluN2 site, glutamatergic receptor)</td>
<td>67 sigma 1 subtype (h)</td>
</tr>
<tr>
<td>28 NMDA receptor (GluN1 site, glutamatergic receptor)</td>
<td>68 sigma 2 subtype (h)</td>
</tr>
<tr>
<td>29 NMDA receptor (PCP site, glutamatergic receptor)</td>
<td>69 sst (somatostatin receptor)</td>
</tr>
<tr>
<td>30 glycine receptor (strychnine-sensitive)</td>
<td>70 VPAC1 (vasoactive intestinal polypeptide receptor 1) (h)</td>
</tr>
<tr>
<td>31 CCR1 (chemokine receptor) (h)</td>
<td>71 V1a (vasopressin receptor) (h)</td>
</tr>
<tr>
<td>32 H1 (histamine receptor) (h)</td>
<td>72 Ca2+ channel (L, verapamil site) (phenylalkylamine)</td>
</tr>
<tr>
<td>33 H2 (histamine receptor) (h)</td>
<td>73 KV channel</td>
</tr>
<tr>
<td>34 H3 (histamine receptor) (h)</td>
<td>74 SKCa channel</td>
</tr>
<tr>
<td>35 MC4 (melanocortin receptor) (h)</td>
<td>75 Na+ channel (site 2)</td>
</tr>
<tr>
<td>36 MT1 (melatonin ML1A receptor) (h)</td>
<td>76 Cl− channel (GABA-gated)</td>
</tr>
<tr>
<td>37 M1 muscarinic receptor (h)</td>
<td>77 norepinephrine transporter (h)</td>
</tr>
<tr>
<td>38 M2 (muscarinic receptor) (h)</td>
<td>78 dopamine transporter (h)</td>
</tr>
<tr>
<td>39 M3 (muscarinic receptor) (h)</td>
<td>79 serotonin transporter (h)</td>
</tr>
<tr>
<td>40 NK1 (tachykinin receptor) (h)</td>
<td></td>
</tr>
</tbody>
</table>

10 μM of PK-209 were tested against all of the above targets, in competition binding assays against agonist or antagonist radioligands.

(h): Recombinant or endogenously expressed human receptors.
6.3. Results

6.3.1. 2-(5-((cyclopentylamino)methyl)pyridin-3-yl)-5-fluorophenol (83)

2-(5-((cyclopentylamino)methyl)pyridin-3-yl)-5-fluorophenol (83) was synthesized in an overall yield of 18%. The compound was analysed by HPLC using method B at 254 nm, and no impurities were observed on UV.

Scheme 1a. Synthesis of precursor 83.

\[ \text{79} \rightarrow \text{80} \rightarrow \text{81} \rightarrow \text{82} \rightarrow \text{83} \]

\text{a Reagents and conditions: (a) Cs}_2\text{CO}_3, \text{PdCl}_2(\text{dpf})-\text{CH}_2\text{Cl}_2, 1,2\text{-dimethoxyethane/ethanol/H}_2\text{O (7/2/1 v/v/v), 12 h, 80 °C}; (b) cyclopentanamine, sodium triacetoxyborohydride, dichloromethane (DCM), 16 h, 0 °C – room temperature; (c) boron tribromide, DCM, 16 h, -78 °C to room temperature (RT).

6.3.2. Radiosynthesis

Several parameters to optimize the radiosynthesis of N-(5-((4-fluoro-2-[\text{11C}]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine \text{[11C]}82 were investigated. All experiments were carried out with 1 mg (3.5 µmol) of precursor 83 and the reaction time was fixed at 5 minutes. Parameters such as type of base, solvent and temperature were varied as listed in Table 2, where yields are decay corrected and either determined by analysing samples taken from the crude reaction mixture (analytical yields) or from the activity at start of synthesis and that in the formulated product (preparative yields).

Aqueous solutions of sodium hydroxide or tetrabutylammonium hydroxide at 85 °C resulted in low yields as determined by analytical HPLC (Method B) independent of the type of solvent used. Anhydrous conditions were investigated by using powdered potassium hydroxide, powdered potassium carbonate or a 60% dispersion of sodium hydride in mineral oil at either room temperature or 85 °C. Application of potassium carbonate as a base resulted in a moderate yield at room temperature when dimethyl
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[11C]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine as a PET tracer for NR2B subunit-
containing NMDA receptors

Sulfoxide was used as a solvent. Applying heat to the reaction further improved the
yield for both solvents, N,N-dimethylformamide and dimethyl sulfoxide, with the
latter being the most favourable solvent.

[11C]82 was prepared in a one-pot synthetic procedure in 49 ± 3% (decay-
corrected) yield, providing 4.1 ± 0.3 GBq of formulated product at the end of synthesis
with a radiochemical purity of >99% and with high specific radioactivity (78 ± 10
GBq/µmol). The stability of [11C]82 (1 mg in 2 mL DMSO or methanol) was tested in a
formulation of 9.6% ethanol with sodium dihydrogen phosphate (7.09 mM) in 0.9%
sodium chloride. After 7 and 19 days the chemical purity of the compound was >96%
(Method B) in both cases.


The methylation reaction proceeded without by-product formation. Small
radioactive peaks were typically observed in the front of the preparative HPLC
chromatogram (method A).

Table 2. Overview of parameters investigated to optimize the radiosynthesis of [11C]82. In all
cases 1 mg (3.5 µmol) of 83 was used and the reaction time was fixed at 5 min.

<table>
<thead>
<tr>
<th>Base</th>
<th>Amount (µmol)</th>
<th>Equivalents</th>
<th>Solvent (300 µL)</th>
<th>Temp.* (°C)</th>
<th>Yield (%)</th>
<th>Yield N #</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH (5M)</td>
<td>25</td>
<td>7.1</td>
<td>DMF</td>
<td>85</td>
<td>10</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>NaOH (5M)</td>
<td>25</td>
<td>7.1</td>
<td>DMSO</td>
<td>85</td>
<td>16</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>KOH</td>
<td>178</td>
<td>50.5</td>
<td>DMSO</td>
<td>85</td>
<td>13</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>TBAOH (2M)</td>
<td>5</td>
<td>1.4</td>
<td>DMSO</td>
<td>85</td>
<td>0.7</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>NaH</td>
<td>50</td>
<td>14.3</td>
<td>DMSO</td>
<td>RT</td>
<td>28</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>NaH</td>
<td>50</td>
<td>14.3</td>
<td>DMSO</td>
<td>85</td>
<td>17</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>NaH</td>
<td>25</td>
<td>7.1</td>
<td>DMF</td>
<td>RT</td>
<td>17</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>NaH</td>
<td>25</td>
<td>7.1</td>
<td>DMF</td>
<td>85</td>
<td>17</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>72</td>
<td>20.6</td>
<td>DMF</td>
<td>RT</td>
<td>16</td>
<td>Analytical 2</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>72</td>
<td>20.6</td>
<td>DMF</td>
<td>85</td>
<td>57</td>
<td>Analytical 2</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>72</td>
<td>20.6</td>
<td>DMSO</td>
<td>RT</td>
<td>49</td>
<td>Analytical 2</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>72</td>
<td>20.6</td>
<td>DMSO</td>
<td>85</td>
<td>74</td>
<td>Preparative 12</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>72</td>
<td>20.6</td>
<td>MeCN</td>
<td>RT</td>
<td>0</td>
<td>Analytical 1</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>72</td>
<td>20.6</td>
<td>MeCN</td>
<td>85</td>
<td>16</td>
<td>Analytical 1</td>
</tr>
</tbody>
</table>

* RT = room temperature.
# number of experiments.
6.3.3. Lipophilicity of $[^{11}\text{C}]82$

The lipophilicity of compound $[^{11}\text{C}]82$ was determined as the LogD$_{7.4}$ in octanol/buffer with a value of LogD$_{7.4} = 2.14 \pm 0.02$ (Mean ± SD).

6.3.4. Affinity

Table 3 shows affinities ($K_i$-values) of 82 and 83 for the NR2B compared with both reference compounds and literature data. The $K_i$-value of 82 for NR2B receptors was $12.4 \pm 2.1$ nM. This affinity is in accordance with previous reports (12 nM) (Table 3). Ifenprodil was used as a reference compound to evaluate the applied assay versus previously published assays. Precursor compound 83 showed poor affinity for the NR2B receptor ($K_i = 1.3 \pm 0.6$ µM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Affinity (nM) measured</th>
<th>Affinity (nM) from literature</th>
<th>Literature reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{11}\text{C}]82$</td>
<td>12.4 ± 2.1*</td>
<td>12 ± 6 a</td>
<td>[17]</td>
</tr>
<tr>
<td>83</td>
<td>1300 ± 600*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[^{3}\text{H}]$ifenprodil$^b$</td>
<td>14.8 ± 5.8</td>
<td>9.1 ± 3.1</td>
<td>[20]</td>
</tr>
<tr>
<td>Ro25-6981</td>
<td>2.3 ± 0.1*</td>
<td>2.9 ± 0.7 c</td>
<td>[21]</td>
</tr>
</tbody>
</table>

* Values are the mean ± standard error of three independent experiments, and were obtained in competition binding experiments against 5 nM $[^{3}\text{H}]$ifenprodil.

a Inhibition of binding of $[^{3}\text{H}]$CP101,606 to rat brain membranes.

b Saturation binding of $[^{3}\text{H}]$ifenprodil to rat brain membranes.

c Saturation binding of $[^{3}\text{H}]$Ro25-6981 to rat brain membranes.

6.3.5. Quantitative in vitro autoradiography results of $[^{11}\text{C}]82$

Brain sections of drug-naive mice were labelled with $[^{11}\text{C}]82$ alone or in the presence of 1 µM Ro25-6981. The distribution of $[^{11}\text{C}]82$ binding sites is shown in Figure 1.

Specific binding (i.e. uptake inhibited by Ro25-6981) was high in areas of cerebral cortex, hippocampus, olfactory tubercle, and striatum, low in thalamus, and absent in sub-thalamic brain regions. Specific binding in the brain regions analysed ranged from 39.6 ± 10.4% in the olfactory tubercle to 3.3 ± 2.1% in the thalamus. High levels of non-specific binding (uptake not inhibited by Ro25-6981) were observed in thalamus and central grey matter.
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Figure 1. Quantitative in vitro autoradiography of [11C]82, showing reduction in binding by 1 μM Ro25-6981(A) and representative images (B).


Brain uptake of [11C]82, measured by tissue counting, was significantly higher at both 5 and 15 min following injection than at 30 and 60 min (Figure 2A; P<0.001, LSD post hoc tests). At 5 min p.i. significantly higher levels of uptake were observed in olfactory tubercle and hypothalamus than in all other brain regions, and at 15 min p.i. these levels were higher than in cerebellum and pons/medulla oblongata (LSD tests). Radioactivity cleared rapidly from the brain and no region-significant differences in [11C]82 accumulation were observed 30 or 60 min following tracer injection (P>0.05, LSD tests). The ratio of mean radioligand uptake in forebrain regions to the cerebellum was higher than 1.9 throughout the study, with the highest ratio of 3.2 ± 0.7 measured at 15 min post-injection, indicating specific uptake. DAR values at 15 min were 1.03 ± 0.37 for olfactory tubercle, 0.64 ± 0.16 for hypothalamus, 0.62 ± 0.22 for entorhinal cortex, 0.33 ± 0.03 for hippocampus, 0.26 ± 0.02 for frontal cortex, 0.25 ± 0.03 for cerebral cortex, 0.24 ± 0.03 for striatum, 0.14 ± 0.02 for pons/medulla oblongata, and 0.14 ± 0.01 for cerebellum. Repeated measures ANOVA confirmed significant main effects of brain region [F(8,81)=2.9, P<0.01] and time [F(3,81)=23.7, P<0.001] on uptake of [11C]82, with nearly significant time x region interaction effects [F(24,81)=2.3, P=0.06].
The organ uptake of $[^{11}C]82$ was higher 5 min post-injection compared with all other time points ($P<0.001$), and was higher in lungs and kidneys than in all other organs examined ($P<0.001$, LSD post-tests; Figure 2B). The highest accumulation of radioactivity was measured in lungs (1.07 ± 0.33) and kidneys (0.68 ± 0.17), 5 min following $[^{11}C]82$ injection. DAR values at 5 min were 0.04 ± 0.01, 0.15 ± 0.05 and 0.19 ± 0.04 for blood, liver and heart, respectively ($P>0.05$ compared with all other time points, LSD post-tests). Activity was rapidly cleared from the periphery, and no significant differences in $[^{11}C]82$ uptake were observed between organs at 30 or 60 min post-injection ($P>0.05$; LSD post-tests). ANOVA confirmed significant main effects
Synthesis and preclinical evaluation of carbon-11 labelled N-((5-(4-fluoro-2-\[\text{11C}\]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine as a PET tracer for NR2B subunit-containing NMDA receptors

of organ \(F(4,45)=13.7, P<0.001\) and time \(F(3,45)= 7.9, P<0.001\), as well as significant organ x time interaction effects \(F(12,45)=3.0, P<0.01\) on the uptake of [\text{11C}]82.

6.3.7. Uptake of [\text{11C}]82 in anaesthetized mice before and after blocking with Ro25-6981

Figure 3 shows distribution (A) and quantification (B) of [\text{11C}]82 uptake in brain sections of control mice, assessed \textit{ex vivo} by autoradiography. Clear uptake in thalamus and hippocampal formation was observed. The highest ratios of region-to-cerebellum uptake were measured in hippocampus (1.76), thalamus (1.66), and dentate gyrus (1.64) of control mice. Pretreatment with Ro25-6981 had inconsistent effects on brain uptake of [\text{11C}]82. In pairs of mice that were administered with identical amounts of radiolabelled compound, Ro25-6981 induced either a mean 22.3 ± 1.9% decrease or a mean 29.4 ± 4.2% increase in radioactivity uptake, across all brain regions analysed. Two-way ANOVA showed no significant main effect of antagonist administration on [\text{11C}]82 uptake (treatment: \(F(1,61)= 0.2, P>0.05\)). Two-tailed t-tests, conducted on individual data sets, confirmed the variable effects of Ro25-6981 administration on brain uptake of [\text{11C}]82. Table summarises conditions and results of 4 independent studies.

![Figure 3](image.png)

**Figure 3.** Distribution (A) and quantification (B) of [\text{11C}]82 uptake in brain sections of control mice, assessed \textit{ex vivo} by autoradiography.
Table 4. Effects of Ro25-6981 on brain uptake of $[^{11}\text{C}]82$, assessed ex vivo by autoradiography in anaesthetized mice.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Condition</th>
<th>Activity injected at $t_0$ (MBq)</th>
<th>Specific activity at $t_0$ (MBq)</th>
<th>Mean change (%)</th>
<th>P value (t-tests)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unblocked</td>
<td>32.23</td>
<td>5.16</td>
<td>-22.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Blocked</td>
<td>30.65</td>
<td>5.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Unblocked</td>
<td>36.72</td>
<td>47.75</td>
<td>-21.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Blocked</td>
<td>35.59</td>
<td>46.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Unblocked</td>
<td>89.09</td>
<td>22.85</td>
<td>33.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Blocked</td>
<td>87.66</td>
<td>22.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Unblocked</td>
<td>124.55</td>
<td>76.24</td>
<td>25.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Blocked</td>
<td>125.05</td>
<td>79.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To visualize the distribution and selectivity of $[^{11}\text{C}]82$ for the NR2B binding site of NMDA receptors, pairs of mice were injected with either saline or Ro25-6981 (10.0 mg/kg, i.p.), 20 min before a tail-vein injection of $[^{11}\text{C}]82$ (n=4 pairs). 15 min after radiotracer injection, mice were killed by cervical dislocation and brains were removed, frozen in liquid nitrogen and processed for quantitative autoradiography. A percent (%) change of control $[^{11}\text{C}]4$ uptake, induced by the administration of Ro25-6981, was obtained for the frontal cortex, parietal cortex, cingulate cortex, striatum, olfactory tubercle, hippocampus, dentate gyrus, occipital cortex, thalamus, hypothalamus and the cerebellum, and averaged across all brain areas analysed.

6.3.8. Metabolite analysis of $[^{11}\text{C}]82$ in anaesthetized mice

$[^{11}\text{C}]82$ was not stable in mouse plasma, as only 6% of the compound was still intact after 1 h (Table 5). 60 min post injection, polar metabolites accounted for approximately 91% of total radioactivity in plasma. At the same time point, radioactivity in brain tissue consisted of about 53% parent compound $[^{11}\text{C}]82$ and 47% polar metabolites (Table 6). No non-polar metabolites were detected in the brain.

Table 5. In vivo metabolite fractions in mouse plasma of $[^{11}\text{C}]82$.

<table>
<thead>
<tr>
<th>time (min)</th>
<th>parent fraction (%) Mean ± SD</th>
<th>polar fraction (%) Mean ± SD</th>
<th>non-polar metabolite (%) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>26 1</td>
<td>72 2</td>
<td>2 1</td>
</tr>
<tr>
<td>15</td>
<td>16 4</td>
<td>81 3</td>
<td>3 1</td>
</tr>
<tr>
<td>30</td>
<td>12 1</td>
<td>86 1</td>
<td>3 1</td>
</tr>
<tr>
<td>60</td>
<td>6 1</td>
<td>91 0</td>
<td>4 0</td>
</tr>
</tbody>
</table>

Table 6. In vivo metabolite fractions in mouse brain of $[^{11}\text{C}]82$.

<table>
<thead>
<tr>
<th>time (min)</th>
<th>parent fraction (%) Mean ± SD</th>
<th>polar fraction (%) Mean ± SD</th>
<th>non-polar metabolite (%) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>91 3</td>
<td>9 3</td>
<td>0 0</td>
</tr>
<tr>
<td>15</td>
<td>84 3</td>
<td>16 3</td>
<td>0 0</td>
</tr>
<tr>
<td>30</td>
<td>70 8</td>
<td>30 8</td>
<td>0 0</td>
</tr>
<tr>
<td>60</td>
<td>53 8</td>
<td>47 8</td>
<td>0 0</td>
</tr>
</tbody>
</table>
6.3.9. Uptake of \[^{11}\text{C}]82\) in non-anaesthetized rats before and after blocking with Ro25-6981

Data on brain uptake of \[^{11}\text{C}]82\) and decrease of uptake of radioactivity following pretreatment with Ro-25-6981 in rats are shown in Figure 4. Pre-treatment with Ro25-6981 resulted in a significant decrease in \[^{11}\text{C}]82\) uptake, both for brain homogenate preparations (Figure 4A) \((P<0.001, \text{Unpaired Student’s t-test})\) and rat

![Figure 4](image)

**Figure 4.** Quantification of \[^{11}\text{C}]82\) uptake in brain homogenates (A) and in brain areas in sections quantified using ex vivo autoradiography (B) of both control and Ro25-6981 treated rats. Figure C shows representative ex vivo autoradiograms of \[^{11}\text{C}]82\) uptake.
brain sections assessed ex vivo by autoradiography (Figure 4B) (treatment: $[F_{(1,20)}=9.6, P<0.01$, Two-way ANOVA). The percentage decrease in $[^{11}C]82$ uptake compared with control was high in occipital cortex (37.2%) and cerebellum (33.9%), moderate in frontal cortex (24.8%), cingulate cortex (22.7%), hippocampus (26.7%) and dentate gyrus (23.4%), and low in striatum (14.9%) and thalamus (7.7%). High levels of non-specific uptake (i.e. not inhibited by Ro25-6981 pretreatment) were observed in the thalamus (Figure 4B). Administration of the NR2B antagonist Ro25-6981 decreased the brain accumulation of $[^{11}C]82$ as shown in ex vivo autoradiograms (Figure 4C).

6.3.10. Pharmacological selectivity profile

At a concentration of 10 µM, compound 82 showed no significant binding for the majority of the targets tested. IC$_{50}$ and K$_i$-values, derived from full concentration-response inhibition curves, for those targets that showed >50% inhibition of specific binding by 10 µM 82, are listed in Table 4.

Table 4. Affinity (IC$_{50}$-, K$_i$-value) and slope of the inhibition curve (Hill coefficient, nH) of 82 for targets showing >50% inhibition in the selectivity screen assays.

<table>
<thead>
<tr>
<th>Target</th>
<th>IC$_{50}$ (M)$^a$</th>
<th>K$_i$ (M)$^b$</th>
<th>nH$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha 1A (h$^d$)</td>
<td>5.6E-06</td>
<td>2.8E-06</td>
<td>1.0</td>
</tr>
<tr>
<td>alpha 2A (h)</td>
<td>5.6E-06</td>
<td>2.5E-06</td>
<td>0.8</td>
</tr>
<tr>
<td>alpha 2B (h)</td>
<td>9.8E-07</td>
<td>6.5E-07</td>
<td>0.7</td>
</tr>
<tr>
<td>alpha 2C (h)</td>
<td>3.4E-06</td>
<td>1.1E-06</td>
<td>1.2</td>
</tr>
<tr>
<td>kappa (KOP)</td>
<td>4.2E-06</td>
<td>2.8E-06</td>
<td>0.9</td>
</tr>
<tr>
<td>mu (MOP) (h)</td>
<td>3.2E-06</td>
<td>1.3E-06</td>
<td>0.8</td>
</tr>
<tr>
<td>5-HT1A (h)</td>
<td>5.8E-07</td>
<td>3.6E-07</td>
<td>0.6</td>
</tr>
<tr>
<td>5-HT2B (h)</td>
<td>3.5E-06</td>
<td>1.7E-06</td>
<td>0.8</td>
</tr>
<tr>
<td>sigma 1 (h)</td>
<td>3.9E-08</td>
<td>2.0E-08</td>
<td>0.9</td>
</tr>
<tr>
<td>sigma 2 (h)</td>
<td>1.3E-06</td>
<td>9.9E-07</td>
<td>0.8</td>
</tr>
<tr>
<td>Na$^+$ channel (site 2)</td>
<td>6.7E-06</td>
<td>6.1E-06</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ is the value that was obtained in the assay used by Cerep (Cerep, Poitiers, France).
$^b$ K$_i$ is the transformation of the value according to the Cheng & Prusoff equation [22].
$^c$ nH is the Hill coefficient.
$^d$ (h): Assays performed on human recombinant or endogenously expressed receptors.
6.4. Discussion

Variation of the type of base used for the radiosynthesis of $[^{11}\text{C}]82$ showed that the basicity of the base influences the reaction yield. Strong bases such as sodium hydride and tetrabutylammonium hydroxide most probably deprotonate the phenolic hydroxyl group as well as the secondary amine. Potassium carbonate deprotonates the acidic phenolic group under mild conditions and is not able to deprotonate the secondary amine even at elevated temperatures.

The assay used to determine the affinity for the ifenprodil binding site of the NR2B unit of the NMDAr gave accurate data, as results obtained in this study were in accordance with previous reports [19,20]. The affinity for the ifenprodil binding site of 82 is about 5 times lower than that observed for Ro25-6981, but equal to that of ifenprodil. A possible metabolite of compound 82, compound 83, has 100 times lower affinity for the NR2B receptor compared with the parent compound. Hence, it is not to be expected, that this metabolite will affect NR2B binding of 82.

The measured LogD$_{7.4}$ value of 2.14 ± 0.02 of $[^{11}\text{C}]82$ is within the optimal range for a compound to cross the blood-brain barrier by passive diffusion, as analysis of experimental data has indicated the optimal range to be between logD values of 1 to 3 [23].

Autoradiography was used to investigate the distribution and selectivity of $[^{11}\text{C}]82$ for the NR2B site of NMDA receptors in vitro. $[^{11}\text{C}]82$ binding was reversed by the selective NR2B antagonist Ro25-6981, showing a regional distribution that is consistent both with the expression pattern of NR2B subunits [24] and the density of $[^3\text{H}]\text{Ro25-6981}$ binding sites in the adult rodent brain [19]. The degree of specific binding, however, was low and dense labelling was observed in brain regions that do not express high levels of NR2B subunit-containing NMDA receptors, most notably in the central grey areas. Due to time constraints dictated by the short half-life of $^{11}\text{C}$ (20.4 min), it was not possible to examine whether this high degree of non-specific binding could be reduced by lowering the concentration of $[^{11}\text{C}]82$ in the incubation mixture and/or increasing the period of incubation, the number of washing steps and the concentration of Ro25-6981 used for displacement. Nevertheless, $[^{11}\text{C}]82$ clearly labelled NR2B subunits on sections of intact brain tissue with preserved integrity, warranting further in vivo characterization of the radioligand.

Biodistribution studies of $[^{11}\text{C}]82$ in anaesthetized mice showed that the compound readily entered the brain, exhibiting heterogeneous uptake in brain regions and fast clearance from both peripheral organs and brain regions. To the best of our knowledge, the 3-fold increase in activity uptake in forebrain regions vs. cerebellum is the highest ratio that has been reported for candidate NR2B site
radiotracers [13]. Despite this favourable profile, pre-treatment with Ro25-6981 had inconsistent effects on the brain uptake of $[^{11}C]82$, either decreasing or increasing its accumulation under identical conditions. The reasons for this finding are unclear, but it is possible that anaesthesia induced alterations in NMDAr function may play a role. Despite the use of an anaesthetic that does not directly act through the NMDAr, there is substantial evidence that fentanyl, for instance, can increase [25], decrease [26], or have no effect [27] on the functional state of NMDArs. Moreover, opioids can simultaneously elicit facilitatory and inhibitory effects on NMDAr function, depending on pre- or postsynaptic localization of the receptor [28]. Midazolam, which stimulates gamma butyric acid-A (GABA-A) receptors and thereby inhibits neuronal activity, might reduce the concentration of endogenous ligands (glutamate, glycine) in the vicinity of the NMDAr, and thereby affect its activation state. Since Ro25-6981 is an ‘activity-dependent’ ligand, showing high affinity predominantly for activated NMDArs [29], it is possible that its potency to block NR2B subunits may have been either increased or diminished in anaesthetized mice, depending on the net effects of anaesthesia on NMDAr function. Similarly, the affinity of ‘cold’ 2,6-disubstituted pyrazines for NR2B subunit-containing receptors has been shown to depend on the functional state of NMDArs [17]. As a result, direct comparisons of $[^{11}C]82$ uptake between control and Ro25-6981 treated mice may have been subject to these variable effects of anaesthesia on NMDAr function.

Metabolite analysis in anaesthetized mice showed that after intravenous injection about 84% and 70% of $[^{11}C]82$ was present in the brain after 15 min and 30 min, respectively. Parent compound $[^{11}C]82$ is present in the brain at a higher level than in plasma, indicating rapid peripheral metabolism with limited amount of metabolites entering the brain. Therefore, metabolism may not be involved in the loss of specific binding of $[^{11}C]82$ [16].

In support of a confounding effect of anaesthesia on blocking the uptake of $[^{11}C]82$, Ro25-6981 administration in non-anaesthetized rats reduced radioactivity uptake compared with control, assessed in both brain homogenate preparations and rat brain sections, indicating that $[^{11}C]82$ can bind to NR2B subunit-containing NMDArs in vivo. Of note, however, high radioactivity uptake, which was inhibited by Ro25-6981 pretreatment, was observed in the cerebellum and pons/medulla, brain regions that are poorly labelled by selective NR2B radioligands in vitro [19,30,31]. Moreover, high levels of residual, non-specific uptake were observed in the thalamus of Ro25-6981 treated rats. These results indicate that, although selective uptake of $[^{11}C]82$ in brain regions enriched with NR2B subunit-containing NMDArs can be demonstrated in vivo, the radiotracer also seems to interact with targets other than the NMDAr. To identify these targets, and in view of the promising results obtained at the radiochemical and
in vivo levels, an exhaustive pharmacological screen against 79 targets was performed. For 78 of the targets examined, 82 had an affinity of >1.1 µM, indicating a high degree of selectivity for the compound. One notable exception, however, was the σ1 site, for which 82 showed an affinity of 20 nM, which is likely to explain the high uptake of the radiotracer in the cerebellum and the pons/medulla regions. Based on these data, it is reasonable to suggest that masking σ receptors with ligands such as haloperidol might increase the ability of [11C]82 to visualize NR2B containing NMDAr even further.

In conclusion, a new NR2B-targeting PET ligand has been developed in high yield. In rodents, [11C]82 readily enters the brain and binds to the NR2B subunit-containing NMDAr. High binding to the sigma-1 site may limit the future application of compound [11C]82 as a PET probe of the NR2B subunit-containing NMDAr. In addition, anaesthesia has an effect on NMDAr function and, therefore, complicates interpretation of preclinical in vivo results. Finally, effects of endogenous compounds on the reduction of observable in vivo specific binding of compound [11C]82 cannot be excluded. Nevertheless, despite these limitations, further studies are warranted to investigate the usefulness of compound [11C]82 as an NR2B PET ligand.

6.5. Acknowledgments

The authors would like to thank Mr Martien Mooijer for his invaluable assistance in the radiosynthesis studies and the BV Cyclotron VU for providing [11C]CO2.

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6.6. References

Synthesis and preclinical evaluation of carbon-11 labelled N-((5-(4-fluoro-2-\[^{11}\text{C}\]methoxyphenyl)pyridin-3-yl)methyl)cyclopentanamine as a PET tracer for NR2B subunit-containing NMDA receptors


Summary, general discussion and future perspectives
7.1. Summary

N-methyl-D-aspartate receptors (NMDAr) are needed for long term potentiation and are thought to play an important role in learning and development. Over-activation of the NMDAr may lead to neural death [1]. Activated NMDAr are receptors in the ‘open’ state e.g. the ion channel is open for Na+, K+ and Ca2+ ions, giving rise to depolarization of the post synaptic membrane. When the receptor is activated ion channel NMDAr antagonists can gain access to the binding site located in the ion channel of the NMDAr [2,3]. The NR2B subunit is involved in modulating functions, such as learning, memory processing and a number of disorders. It is suggested that activation of the NR2B subunit initiates apoptotic signaling cascades and promotes neural death [4]. As such, radiolabelled ligands in combination with molecular imaging techniques such as positron emission tomography (PET) may be valuable tools to assess the status of regional NMDAr activation in vivo [5].

This thesis describes the development of novel carbon-11 and fluoro-18 NMDAr radiotracers.

Chapter 2 describes the development of a series of novel di- and tri-N-substituted diarylguanidines (Figure 1). Known from structure activity relationship studies was that a meta substituted aryl group at R1 was favourable. Either R2 or R3 should be methylated to reduce affinity for the sigma-receptor. The other guanidine aryl group enhances the potency the most when the aryl was substituted with either being R4 a chloro or bromo and R5 being a thiomethyl moiety. Little was known about the effects of the length of the spacer (n) between the guanidine core and R1.

![Figure 1. General structure of N,N'-di and tri-substituted aryl guanidines.](image)

Three series of compounds were synthesized and their binding affinity to the ion channel of the NMDAr was assessed. Substituted 3-methoxy-aryl (R1) and methylation of R2 whilst R3 is hydrogen showed highest affinities. Introducing a spacer (methyl or ethyl) decreased the affinity with orders of 10^2 to 10^6 compared to those without spacer. Three novel compounds, 15 (difluoromethoxy aryl, 10.2 nM), 16 (trifluoromethoxy aryl, 11.7 nM) and 37 (fluoromethoxy aryl, 18.4 nM) showed high affinity. These ligands were radiolabelled with either carbon-11 or fluorine-18. Ligands [11C]15 and [11C]16 were obtained in 26 and 15% decay corrected radiochemical yield and high (> 170 GBq·µmol⁻¹) specific activity within 38 minutes.
Ligand $[^{18}F]37$ was obtained in 22% yield with a specific activity of 53 GBq-µmol$^{-1}$ in 92 minutes. Lipophilicities, expressed as logD$_{oct,7.4}$, were 1.76, 2.76 and 1.45, respectively. Ligands $[^{11}C]15$ and $[^{18}F]37$ were evaluated ex vivo in B6C3 mice. Biodistribution studies showed higher uptake in forebrain regions compared with cerebellum. Pre-treatment with MK-801 (0.6 mg·kg$^{-1}$) slightly decreased uptake in NMDAr-specific regions for $[^{18}F]37$, but not for $[^{11}C]15$. After 60 minutes the amount of intact tracer was 54 and 70% for $[^{11}C]15$ and $[^{18}F]37$, respectively.

In chapter 3 a series of novel amine guanidine derivatives is presented based on the optimal ligand structure as found in chapter 2 (Figure 2). Three ligands, 59 (dimethylamino aryl, 1.35 nM), 61 (methylamino aryl, 19.1 nM) and 64 (fluoroethyl(methyl)amino aryl, 4.81 nM), showed favourable affinities. These were all short alkyl substituted benzyl amines that were labelled with carbon-11 (59, 61) or fluorine-18 (64). Ligands $[^{11}C]59$ and $[^{11}C]61$ were obtained in 31 and 36% decay corrected radiochemical yield, respectively. The specific activity was higher than 105 GBq·µmol$^{-1}$ and total synthesis time was 22 minutes. Ligand $[^{18}F]64$ was synthesized in 9% decay corrected radiochemical yield with a specific activity of 78 GBq·µmol$^{-1}$. Lipophilicity, expressed as logD$_{oct,7.4}$, were 1.62, 1.24 and 1.51 for $[^{11}C]59$, $[^{11}C]61$ and $[^{18}F]64$, respectively.

Figure 2. General structure of $N,N'$-tri-substituted aryl guanidines.

Ligands $[^{11}C]59$, $[^{11}C]61$ and $[^{18}F]64$ were evaluated ex vivo in male Wistar rats. Biodistribution showed good uptake in the brain, but following pre-treatment with MK-801 (0.6 mg·kg$^{-1}$) none showed a reduction in uptake. Metabolite studies indicated that all three ligands suffered from fast metabolism and that metabolites did enter the brain as more than 55% of radioactivity in the brain after 45 minutes was due to radiolabeled metabolites. This uptake could conceal a possible specific signal. Despite high affinity for the NMDAr, these ligands did not show appropriate characteristics for use as a PET tracer.

Chapter 4 describes the synthesis of $[^{11}C]78b$, an analogue of $[^{18}F]37$, called $[^{18}F]PK-209$. Here, the fluoromethoxy moiety was substituted with a vinylic moiety. The purpose of this substitution was the assumption that metabolic stability of $[^{18}F]PK-209$ would improve by preventing metabolic oxidation of the fluoromethoxy moiety. The affinity of $78b$ was 18.6 nM. After labelling with carbon-11, $[^{11}C]78b$ was obtained in 20% decay corrected radiochemical yield with a specific activity of 112 GBq·µmol$^{-1}$. The logD$_{oct,7.4}$ value was 2.62. Ex vivo biodistribution studies in male
Wistar rats showed higher uptake of $[^{11}C]78b$ in the forebrain regions compared with cerebellum. Pre-treatment with MK-801 (0.6 mg·kg$^{-1}$) decreased overall brain uptake, but not in a region specific manner. Metabolism was compared between $[^{11}C]$GMOM, $[^{18}F]$PK-209 and $[^{11}C]78b$, and 45 minutes after injection 78, 90 and 87%, respectively, of the radioactivity in the brain was due to parent compound while in plasma 26-31% of the radioactivity was due to parent compound. Although more parent compound was present in plasma for $[^{11}C]78b$ than for $[^{11}C]$GMOM and $[^{18}F]$PK-209, this increase was not significant. At 45 minutes, in the brain, significantly more parent compound of $[^{18}F]$PK-209 and $[^{11}C]78b$ was measured compared with $[^{11}C]5$.

In chapter 5 the preclinical evaluation of the most promising NMDA radiotracer developed in chapter 2, i.e. $[^{18}F]$PK-209 ($[^{18}F]37$), is described (Figure 3). The selectivity profile of PK-209 showed that it was at least 50-fold more selective for NMDArs than for other targets examined. $[^{18}F]$PK-209 was further evaluated using dynamic PET scanning (120 minutes) in rhesus monkeys under baseline conditions and after pre-treatment with MK-801 (0.3 mg·kg$^{-1}$). $[^{18}F]$PK-209 entered the brain rapidly, showing a fairly uniform distribution in the brain areas examined. In two out of three subjects a reduced parametric volume-of-distribution ($V_T$) was found compared with baseline conditions. The third subject showed an increase of $V_T$. The reason for the increase in the third subject is not known, although it could be related to the administration of a partial µ-opiod receptor agonist that was given to the monkey. It is known that this agonist can increase the function of NMDArs. $[^{18}F]$PK-209 showed a high rate of metabolism in vivo. However, Logan plots supported the initial finding that no significant accumulation of $[^{18}F]$PK-209 derived metabolites occurred in the brain.

![Figure 3. $[^{18}F]$PK-209 ($[^{18}F]37$), the NMDAr tracer that was evaluated preclinically.](image)

In chapter 6 the synthesis and preclinical evaluation of an NR2B-subunit radiotracer is described. Compound $[^{11}C]82$ was labelled with carbon-11 in 49% decay corrected radiochemical yield and a specific activity of 78 GBq·µmol$^{-1}$. The affinity of 82 towards the NR2B-subunit of the NMDAr was 12.4 nM, the lipophilicity ($\log D_{oct7.4}$) was 2.14. Ex vivo radiodistribution of $[^{11}C]82$ in male B6C3F1/J rats showed that it readily enters the brain with a 3-fold increase in uptake in forebrain regions compared with cerebellum. Pre-treatment with Ro 25-6981 (10.0 mg·kg$^{-1}$) led to inconsistent effects on cerebral uptake of $[^{11}C]82$, with either decreases and
increases under identical conditions. Metabolite analysis showed that after 30 minutes 70% of the radioactivity in the brain was due to parent compound. A selectivity screen over 79 targets revealed that 82 also had a significant affinity for the sigma-1 receptor of 20 nM. This could hamper its use as an NMDA NR2B-subunit PET tracer.

7.2. General discussion

The work described in this thesis concerns the development of radiotracers to image the NMDAr using PET. Lead structure identification showed that comprehensive structure relationship studies had been published based on compounds bearing a diaryl-guanidine core, with some compounds showing affinity in the nanomolar range (< 10 nM) for the NMDAr. A few, e.g. CNS-1261, GMOM and CNS-5161 had already been radiolabelled and evaluated in vivo, but results indicated that these radiotracers were not ideal as PET NMDAr tracers. Since those results were not encouraging, there appeared to be room for improvement.

Chapter 2, 3 and 4 describe compounds based on the diaryl-guanidine core. Seven new compounds (Chapter 2: 15, 16, 37; Chapter 3: 59, 61, 64; Chapter 4: 78b) from the various series synthesized showed high affinity (< 20 nM, [3H]MK-801) towards the ion channel of the NMDAr. It is interesting to note that the competition binding assay developed in-house provided lower affinities for all known compounds than those reported previously (CNS-1261: 4.2 vs. 8.7 nM, GMOM: 5.2 vs. 20.7 nM, and CNS-5161: 1.9 vs. 3.5 nM) [6]. Many factors can have an effect on the results of the competition binding assay. The most important ones are the concentrations of glutamate and glycine. Previous studies have used 10 mM concentrations of both the agonist glutamate and the co-agonist glycine, which represent supra-saturating conditions [7]. In the present thesis, the assay was performed using 1 µM concentrations of both. To confirm our affinity results, GMOM and 37 were screened externally and confirmed affinity values of the binding assay.

A common attribute of the compounds exhibiting high affinity is the presence of a short alkyl moiety substituted on either hydroxyl (78b, 15, 16, and 37) or amine (59, 61, and 64) functionality at the meta position of the aryl group. This indicates that small structural alterations at this position are tolerated by the ion channel of the NMDAr without losing affinity. Furthermore, as shown in chapter 2, an N-methyl group on the guanidine adjacent to the meta substituted aryl group has a positive effect on the binding affinity towards the NMDAr. A further advantage is that this methyl moiety suppresses the affinity towards the sigma receptor, resulting in better selectivity.
Five compounds (78b, 15, 16, 59 and 61) were labelled with carbon-11 using straightforward methylation reactions, resulting in radiochemical yields ranging from 15 to 31% and specific activities ranging from 105 to 226 GBq·µmol⁻¹. Two compounds (37 and 64) were labelled with fluorine-18, either via [¹⁸F]FCH₂Br or via direct nucleophilic substitution in 22 and 9% radiochemical yield with specific activities of 53 and 78 GBq·µmol⁻¹, respectively.

Radiotracers ([¹¹C]78b, [¹¹C]15, [¹⁸F]37, [¹¹C]59, [¹¹C]61 and [¹⁸F]64) were evaluated ex vivo. Compounds [¹¹C]15 and [¹⁸F]37 were evaluated in anaesthetized B6C3F1/J mice, whilst the remaining compounds were evaluated in non-anaesthetized male Wistar rats to avoid any confounding effect of anaesthesia on NMDAr binding. A lack of specific binding has been noted for several ion channel tracers due to anaesthesia, i.e. isoflurane is known to deactivate NMDAr channels [5]. Biodistribution studies showed that all radiotracers passed the blood brain barrier. This was expected based on their lipophilicity with Log₂oct,7.4 values ranging from 1.24 to 2.62. In nearly all cases the lowest uptake was seen in the cerebellum and the highest in forebrain regions, i.e. in line with the distribution of NMDAr in the brain. In contrast, the uptake of [¹⁸F]37 was uniform across the brain and there was no washout of radioactivity from the brain. All radiotracers showed uptake in the lungs followed by kidneys, liver and heart. Pre-treatment with MK-801 led to inconsistent results. Ex vivo autoradiography showed that, after pre-treatment, uptake of [¹¹C]15 increased, whilst uptake of [¹⁸F]37 decreased in mice. Ex vivo biodistribution in rats showed an increase after pre-treatment for [¹¹C]59, [¹¹C]61 and [¹⁸F]64. On the other hand, [¹¹C]78b showed a decrease in overall brain uptake after pre-treatment, but a t-test showed no region specific differences after MK-801 administration. The reason for increased uptake after pre-treatment with MK-801 is unclear. Reasons for this effect could be that there is a release of glutamate from the brain after MK-801 administration or an increase in blood flow [8]. It is also possible that after pre-treatment the peripheral NMDAr receptors are occupied by MK-801 and thus more ligand is available to the brain. However, ex vivo results do not show a significant lower uptake in NMDAr rich organs such as the lungs. [¹⁸F]37 was also evaluated in male rhesus monkeys. Pre-treatment with MK-801 did decrease the VT in two out of three subjects and showed an increase in the third subject. Subject three was administered with the partial µ-opioid receptor agonist buprenorphine and are known to increase the function of the NMDAr [9]. These results suggest that one can speculate that the increased uptake in subject three may actually reflect the ability to quantify alterations in the functional state of the NMDArs. However, this should be done with caution since the actual reason for increase VT of subject three is unknown.
An important characteristic of a PET tracer is its metabolism, as radiolabelled metabolites may hamper quantification of PET signals. Ligands $[^{11}\text{C}]15$ and $[^{18}\text{F}]37$ were evaluated in mice and both show rapid appearance of metabolites in plasma, although less metabolites were seen in the brain. Compounds $[^{11}\text{C}]59$, $[^{11}\text{C}]61$ and $[^{18}\text{F}]64$ were evaluated in rat and all showed rapid metabolism. Both polar and non-polar metabolites were able to enter the brain. Metabolism of $[^{18}\text{F}]37$ and $[^{11}\text{C}]5$ and $[^{11}\text{C}]78b$ was assessed in rats. For $[^{18}\text{F}]37$ a slower rate of metabolism was observed than in mice. Whereas in mice after 30 minutes 76% of radioactivity in the brain was due to parent compound, in rat after 45 minutes 90% was accountable to $[^{18}\text{F}]37$. The metabolism rate of $[^{11}\text{C}]78b$ was slightly lower than for $[^{18}\text{F}]37$ (87%) but not significant. Both were less vulnerable for metabolism than $[^{11}\text{C}]5$ (78%).

The ligands discussed above are all targeting the ion channel of the NMDAr, this is only possible when the NMDAr is the so called ‘open’ state. These ligands are therefore useful to image and quantify the amount of open (active) NMDAr. To quantify the total amount of NMDAr in the brain another class of ligands should be used targeting other binding sites on the NMDAr, such as the NR2B site. The NR2B targeting ligand $[^{11}\text{C}]82$ was developed and evaluated ex vivo in anaesthetized mice. Ligand $[^{11}\text{C}]82$ readily entered the brain and did bind to the NR2B subunit with a 3-fold ratio in forebrain regions over cerebellum. Binding of $[^{11}\text{C}]82$ was reversed by the selective NR2B antagonist Ro 25-6981. However, pre-treatment with Ro 25-6981 showed inconsistent effects, possible due to effects of Ro 25-6981 itself on the functional state of the NMDAr. Interactions of local anesthetics and the NMDAr are known and are demonstrated in in-vitro models and volunteers [10]. Administration of $[^{11}\text{C}]82$ in non-anaesthetized rats showed, after Ro 25-6981 treatment, a reduced uptake compared with control. It should be noted that $[^{11}\text{C}]82$ also seems to interact with other targets, which is confirmed by a selectivity screen showing affinity of 20 nM towards the sigma-1 receptor.

### 7.3. Future perspectives

Anaesthesia may have an effect on NMDAr status, but the exact nature of the final effect it is not yet clear. For example, there are contradictory reports on the effects of fentanyl, where increases, decreases or no effects on the state of the NMDAr have been reported [11-13]. Therefore, in the development of successful tracers for the NMDAr one should be careful in interpreting preclinical results and pursue PET imaging in healthy volunteers as soon as possible. Preliminary results of the human test / retest study using $[^{18}\text{F}]PK209$ showed that binding of $[^{18}\text{F}]PK209$ is insufficiently reproducible to quantify the NMDAr. This could be due to the number of ‘activated’
open NMDAr are most likely different at different time points. These results do not indicate that \(^{18}\text{F}\)PK209 is not capable to bind to the NDMAr, but an underlying mechanism may have a larger influence than desirable. Therefore further research to understand this mechanism is needed.

All radiotracers developed in this thesis suffer from metabolism to a variable degree. It was shown, however, that substitution of the hydrogen in the methoxy moiety prevents its metabolism. It would be interesting to investigate, whether modification of the other moieties that are susceptible to metabolism, i.e. thiomethyl and N-methyl, has a positive effect on the stability of the ligand \textit{in vivo} without lowering the affinity towards the NMDAr. Ligand \(^{11}\text{C}\)78b would be the ideal starting point for this improvement as this tracer showed the slowest metabolic degradation of tracers presented in this thesis.

Another interesting tracer to investigate more is the presented NR2B subunit tracer. Preliminary data of \(^{11}\text{C}\)82 shows that specific binding \textit{in vivo} in rhesus monkeys could not be visualized, which could be due to the relatively high binding affinity towards the sigma-1 receptor. As suggested in chapter 6, masking sigma receptors with ligands such as haloperidol might increase the ability of \(^{11}\text{C}\)82 to visualize the NR2B receptor subunits. Structural there are opportunities to alter the molecule to improve the affinity towards the NMDAr. Potential alterations are likely to be found the cyclopentanamine moiety of the tracer [14,15]. Beneficial would be a decreased affinity for other targets like the sigma receptors.
7.4. References


Curriculum vitae

Pieter Jacob Klein was born on October 8, 1979 in Utrecht, the Netherlands. After
finishing secondary school (HAVO) at OSG Echnaton in Almere, he obtained his
Bachelor of Applied Sciences (ingenieur) in the field of organic chemistry at the
University of Applied Sciences Utrecht. During the internship at the Radionuclide
Center VU his interest in radio chemistry was aroused. In 2001 he started as a
research technician under the supervision of dr. A.D. Windorst. In 2009 he took the
opportunity to start a PhD project at the VU University Medical Center Amsterdam
under the supervision of prof. dr. A.D. Windhorst, prof dr. B.N.M. van Berkel and prof.
dr. A.A. Lammertsma, on the development of radiopharmaceuticals for the NMDA
receptor. The results of which are described in this thesis. Following completion of the
practical part of his PhD work, he joined TEVA Pharmachemie as an analytical
scientist. In 2017 he worked on a small project at the Otolaryngology/Head & Neck
Surgery at the VU University Medical Center Amsterdam. After that he moved to
Dr.Reddy’s Research and Development Leiden as a scientist analytical method
development.
List of publications


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