

Radiosynthesis and in vivo evaluation of a series of substituted ^{11}C -phenethylamines as 5-HT $_2\text{A}$ agonist PET tracers

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Abstract

Purpose Positron emission tomography (PET) imaging of serotonin 2A (5-HT $_2\text{A}$) receptors with agonist tracers holds promise for the selective labelling of 5-HT $_2\text{A}$ receptors in their high-affinity state. We have previously validated [^{11}C]Cimbi-5 and found that it is a 5-HT $_2\text{A}$ receptor agonist PET tracer. In an attempt to further optimize the target-to-background binding ratio, we modified the chemical structure of the phenethylamine backbone and carbon-11 labelling site of [^{11}C]Cimbi-5 in different ways. Here, we present the in vivo validation of nine novel 5-HT $_2\text{A}$ receptor agonist PET tracers in the pig brain. **Methods** Each radiotracer was injected intravenously into anaesthetized Danish Landrace pigs, and the pigs were

subsequently scanned for 90 min in a high-resolution research tomography scanner. To evaluate 5-HT $_2\text{A}$ receptor binding, cortical nondisplaceable binding potentials (BP $_{\text{ND}}$) were calculated using the simplified reference tissue model with the cerebellum as a reference region.

Results After intravenous injection, all compounds entered the brain and distributed preferentially into the cortical areas, in accordance with the known 5-HT $_2\text{A}$ receptor distribution. The largest target-to-background binding ratio was found for [^{11}C]Cimbi-36 which also had a high brain uptake compared to its analogues. The cortical binding of [^{11}C]Cimbi-36 was decreased by pretreatment with ketanserin, supporting 5-HT $_2\text{A}$ receptor selectivity in vivo. [^{11}C]Cimbi-82 and [^{11}C]Cimbi-21 showed lower cortical BP $_{\text{ND}}$, while [^{11}C]Cimbi-27, [^{11}C]Cimbi-29, [^{11}C]Cimbi-31 and [^{11}C]Cimbi-88 gave rise to cortical BP $_{\text{ND}}$ similar to that of [^{11}C]Cimbi-5.

Conclusion [^{11}C]Cimbi-36 is currently the most promising candidate for investigation of 5-HT $_2\text{A}$ receptor agonist binding in the living human brain with PET.

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Introduction

The serotonin 2A (5-HT $_2\text{A}$) receptors are implicated in the pathophysiology of human diseases such as depression and schizophrenia, and 5-HT $_2\text{A}$ receptor stimulation is responsible for the hallucinogenic effects of recreational drugs such as lysergic acid diethylamide (LSD) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) [1], whilst the therapeutic effects of atypical antipsychotics

can be attributed to the antagonistic effects on these receptors [2]. Positron emission tomography (PET) imaging of cerebral 5-HT_{2A} receptors is used to characterize the serotonergic receptor system in disease states, and PET imaging can also be used to measure receptor occupancy by therapeutic drugs, e.g. antipsychotics.

Currently, only antagonistic PET ligands such as [¹⁸F]altanserin [3] and [¹¹C]MDL100907 [4] are available to selectively map and quantify 5-HT_{2A} receptor binding in the human brain. However, whereas 5-HT_{2A} receptor antagonists bind to the total pool of receptors, 5-HT_{2A} receptor agonists selectively bind to receptors in their high-affinity state [5, 6]. Thus, a 5-HT_{2A} receptor agonist PET tracer would ideally bind only to 5-HT_{2A} receptors in their functional state. Alterations in agonist binding measured in vivo with PET may be more relevant for assessing dysfunction in the 5-HT_{2A} receptors in specific patient or population groups. Furthermore, since a large fraction of the 5-HT_{2A} receptors are intracellularly localized [7, 8], combining measurements with antagonist and agonist PET tracers would enable in vivo determination of the ratio of the high-affinity, membrane-bound and active receptors to the low-affinity, inactive and intracellular receptors [9]. Thus, quantification of functionally active 5-HT_{2A} receptors in vivo using an agonist PET tracer is hypothesized to be superior to antagonist measurements of total pool of 5-HT_{2A} receptors for studying alterations in receptor function in human diseases such as depression.

In terms of chemical structure, 5-HT_{2A} receptor agonists fall into three classes: tryptamines, ergolines and phenethylamines. Recently, several *N*-benzyl-substituted phenethylamines have been described as superpotent and selective 5-HT_{2A} receptor agonists with EC₅₀ values up to 27-fold lower than that of 5-HT itself [10]. One of these compounds, 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine (25I-NBOMe, Cimbi-5), was recently tritiated [11], and we have also evaluated [¹¹C]Cimbi-5 as a 5-HT_{2A} receptor agonist PET tracer [12].

Dopamine D₂ receptor agonist radiotracers are superior to antagonist radiotracers for measuring dopamine release in vivo in humans [13], monkeys [14] and mice [9], and since several studies have failed to demonstrate that 5-HT_{2A} receptor antagonist PET tracers are displaceable by elevated levels of endogenous 5-HT [15], it may well be that 5-HT_{2A} receptor agonists would be more prone to displacement by competition with endogenously released 5-HT. Monitoring the release of endogenous 5-HT is highly relevant in relation to human diseases such as depression and Alzheimer's disease which involve dysfunction of the 5-HT system.

Here, we present the synthesis and evaluation of a series of ¹¹C-phenethylamines structurally related to the previously validated lead compound [¹¹C]Cimbi-5. The agonistic properties of the compounds were ascertained in vitro by

phosphoinositide (PI) hydrolysis assays and binding assays. To test the suitability of the compounds as PET tracers in vivo, all substituted phenethylamines were labelled with carbon-11, and cerebral uptake, distribution and displacement were investigated in pigs after intravenous (i.v.) injection of PET tracer.

Materials and methods

Chemical synthesis

Synthesis of precursors and radiochemical labelling are summarized in Fig. 1; bold numbers refer to this figure. Experimental conditions, synthesis routes for the precursors, and NMR data for previously unpublished intermediates can be found in the [Supplementary material](#). The precursors for the radiolabelling were, with the exception of **14**, synthesized in two steps from their parent phenethylamines. Reductive amination with the appropriate aldehydes followed by selective Boc-protection of the secondary amines gave the labelling precursors. The syntheses of the parent phenethylamines **1** [16], **2** [16], **3** [17] and **4** [18] have been described elsewhere. The synthesis of **5** was in four steps from 1,4-diiodo-2,5-dimethoxybenzene, and the synthesis of **14** was in 3 steps from 2-(2-isopropoxy-5-methoxyphenyl)ethanamine [11] as described in the [Supplementary material](#). The synthesis of reference compounds except Cimbi-82 have been reported elsewhere [10, 16]. The synthesis of Cimbi-82 is described in the [Supplementary material](#). The lipophilicity of all PET tracers (cLogD_{7.4}) was calculated using CSLogD (ChemSilico).

Radiochemical synthesis of ¹¹C-phenethylamines

Radiochemical labelling of all PET tracers is summarized in Fig. 1. All radiolabelled compounds except [¹¹C]Cimbi-88 were prepared as follows. [¹¹C]Methyl triflate was collected in a solution of 0.3–0.4 mg labelling precursor (see Fig. 1) in a mixture of acetonitrile (200 µl) and acetone (100 µl) containing 2 µl 2 M NaOH at room temperature, and the solution was subsequently heated for 30 s at 40°C. Subsequently, 250 µl of a 1:1 mixture of trifluoroacetic acid/CH₃CN was added and the mixture heated at 80°C for 5 min. After neutralization with 750 µl 2 M NaOH and dilution with about 4 ml citrate buffer (25 mM, pH 4.7), the reaction mixture was purified by HPLC (Phenomenex Luna C18(2), 250×10 mm column; 40/60 acetonitrile/25 mM citrate buffer pH 4.7, flow rate 6 ml/min).

The HPLC fraction containing the product was collected in a flask containing 50 ml 0.1% ascorbic acid. This solution was then passed through a C18 SepPak light column which had been preconditioned with 10 ml ethanol

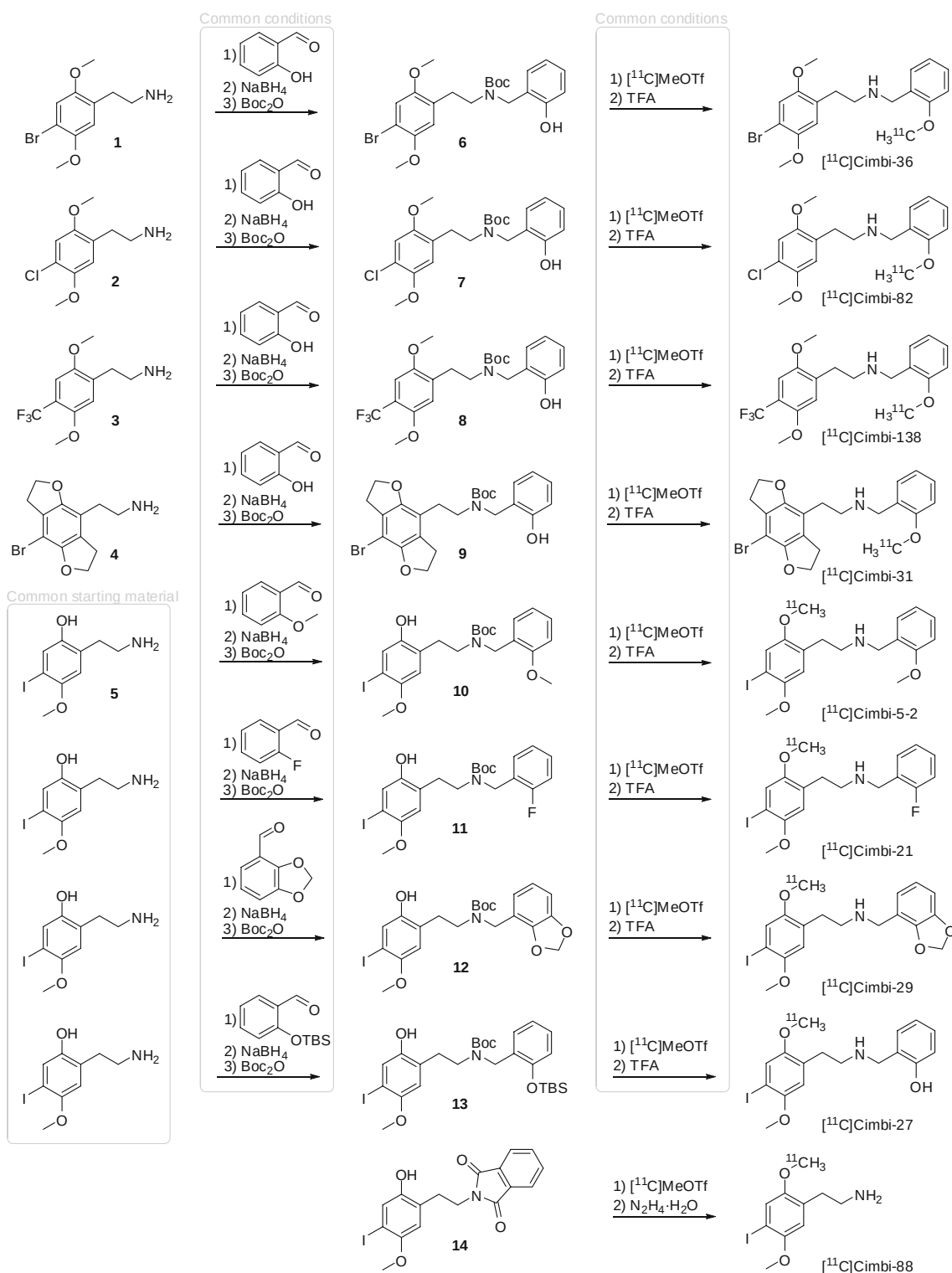


Fig. 1 Chemical synthesis of precursor compounds and radiochemical preparation of PET tracers. For synthesis specifications, refer to the [Supplementary material](#)

followed by 20 ml 0.1% ascorbic acid. The column was first flushed with 3 ml sterile water, then the trapped activity was eluted with 3 ml ethanol followed by 3 ml 0.1% ascorbic acid into a 20-ml vial containing 9 ml

phosphate buffer (100 mM, pH 7) giving a 15 ml solution of the labelled product. The total synthesis time was 40–50 min. Analysis to determine radiochemical purity and specific radioactivity was performed using HPLC with

online UV and radiodetection (Luna C18(2) 4.6×150 mm column; eluent 40/60 acetonitrile/25 mM citrate buffer pH 4.7; flow rate 2 ml/min, wavelength 300 nm).

[¹¹C]Cimbi-88 was synthesized in an analogous manner to the above except that the deprotection step was performed by addition of hydrazine monohydrate (300 µl) and heating at 120°C for 3 min. The solution was neutralized with 2 ml 3 M HCl, diluted with 2.5 ml citrate buffer (25 mM, pH 4.7) and purified as described above. Radiochemical purity of all radiolabelled compounds was >95% and specific activities at the end of synthesis varied from 14 to 605 GBq/µmol.

In vitro binding

Competition binding experiments against [³H]MDL100907 were performed in a NIH-3T3 cell line (GF62) stably transfected with the rat 5-HT_{2A} receptor as previously described [19] using 0.2 nM [³H]MDL100907 as the radioactive competitor. Eight different concentrations of ligand (from 1 µM to 1 pM) in a total of 1 ml buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.01% ascorbic acid, pH 7.4) including cell homogenate were tested. Nonspecific binding was determined with 1 µM ketanserin. The incubation was terminated after 1 h by filtration using a 24-channel 300-ml cell harvester (Brandel). Tris-HCl buffer was used for washing, and the samples were filtered through a Whatman GF/B filter. The filters were soaked with 1% polyethylenimine prior to filtration in order to reduce and stabilize nonspecific binding to the filters. Radioactive concentrations were determined with a scintillation counter (Packard Instruments), and the *K_i* values were calculated based on percent binding inhibition of the radioactive ligand. Furthermore, values of *K_i* against various neuroreceptors were provided by the Psychoactive Drug Screening Program (PDSP; for experimental details refer to the PDSP website <http://pdsp.med.unc.edu/>).

PI hydrolysis assay

GF62 cells (1.5×10⁶ cells/ml) were cultured overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate (Sigma), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in an atmosphere containing 5% CO₂. Subsequently, cells were incubated overnight with 4 µCi/well of myo-(1,2)-[³H]-inositol (Amersham) in labelling medium (inositol-free DMEM containing 10% FBS and penicillin/streptomycin). The cells were then washed once with incubation buffer (20 mM HEPES, pH 7.4; 20 µM LiCl, 1 mM MgCl₂, 1 mM CaCl₂) and incubated at 37°C in the same buffer for 30 min in the presence or absence of

1 µM ketanserin. The solutions were removed and test compounds (10 µM to 0.1 pM) or 5-HT (10 µM) were added to the wells for 30 min at 37°C. The formed inositol phosphates were then extracted with 10 mM ice-cold formic acid for 30 min at 4°C. The supernatants were transferred to AG 1-X8 anion exchange resin columns (Bio-Rad) and eluted into Ultima-FLO AF scintillation liquid (Packard) with 2 M ammonium formate/0.1 M formic acid. Accumulated [³H]inositol phosphates were measured with a Tri-Carb 2900TR liquid scintillation counter (Packard Instruments) after 1 h incubation at room temperature.

Animal procedures

Ten female Danish Landrace pigs were used; their mean weight was 20.1±3.8 kg. After arrival, the animals were housed under standard conditions and were allowed to acclimatize for 1 week before scanning. To minimize stress, the animals were provided with straw bedding and environmental enrichment in the form of plastic balls and metal chains. On the scanning day, pigs were tranquilized by intramuscular injection of 0.5 mg/kg midazolam. Anaesthesia was induced by intramuscular injection of a Zoletil veterinary mixture (1.25 mg/kg tiletamin, 1.25 mg/kg zolazepam and 0.5 mg/kg midazolam; Virbac Animal Health). Following induction, anaesthesia was maintained by i.v. infusion of 10 mg/kg propofol per hour (B. Braun Melsugen). During anaesthesia, animals were endotracheally intubated and ventilated (volume 250 ml, frequency 15 per min). Venous access was obtained through two Venflon cannulas (Becton Dickinson) in the peripheral milk veins, and an arterial line for blood sampling was inserted into the femoral artery via a minor incision. Vital signs including blood pressure, temperature and heart rate were monitored throughout the duration of the PET scanning. Immediately after scanning, animals were killed by i.v. injection of pentobarbital/lidocaine. All animal procedures were approved by the Danish Council for Animal Ethics (Journal No. 2006/561-1155).

PET scanning protocol

All PET tracers were evaluated with a single PET scan. On the basis of the pharmacokinetic properties, the best candidate was selected for further investigation including a blocking study in vivo and an examination of metabolites in pig brain tissue. All PET tracers were given as an i.v. bolus injection, and the pigs were subsequently scanned for 90 min in list mode with a high-resolution research tomography (HRRT) scanner (Siemens). Scanning was started at the time of injection (*t*=0). The injected radioactivities and specific radioactivities at the time of injection are given in Table 2. In all pigs, arterial whole blood

samples were taken throughout the entire scan. During the first 15 min after injection, radioactivity in whole blood was continuously measured using an Allogg ABSS auto-sampler (Allogg Technology) counting coincidences in a lead-shielded detector. Concurrently, blood samples were manually drawn at 2.5, 5, 10, 20, 30, 50, 70 and 90 min, and radioactivity in whole blood and plasma was measured using a well counter (Cobra 5003; Packard Instruments) that was cross-calibrated to the HRRT scanner and to the autosampler. Also, radiolabelled parent compound and metabolites were measured in plasma as described below.

To test the displaceability of [^{11}C]Cimbi-36 by a known 5-HT_{2A} receptor antagonist *in vivo*, ketanserin tartrate (10 mg/kg *i.v.*; Sigma no. S006) was given after a [^{11}C]Cimbi-36 baseline scan 30 min prior to a second scan using the same PET protocol. In these two scans, injected radioactivities were 553 MBq and 590 MBq in the baseline and blocked scan, whereas the specific radioactivities at the time of injection were 175.4 GBq/ μmol and 257.0 GBq/ μmol .

Quantification of PET data

Data from a 90-min HRRT list mode PET scans were reconstructed using a standard iterative method as previously reported [20] (OSEM3D-OP with point spread function, ten iterations, 16 subsets) into 38 dynamic frames of increasing length (6 $\times$ 10, 6 $\times$ 20, 4 $\times$ 30, 9 $\times$ 60, 3 $\times$ 120, 6 $\times$ 300, 4 $\times$ 600 s). Images consisted of 207 planes of 256 $\times$ 256 voxels of 1.22 $\times$ 1.22 $\times$ 1.22 mm. A summed image of all counts in the 90-min scan time for each pig was reconstructed and used for coregistration to a standardized MRI-based statistical atlas of the Danish Landrace pig brain, similar to that previously reported for the Göttingen minipig [21] using the program Register as previously described [22]. Hereafter, the activity in volumes of interest (VOI), including the cerebellum, cortex (defined in the MRI-based atlas as the entire cortical grey matter), hippocampus, caudate putamen, putamen, dorsal and ventral thalamus, and lateral ventricle, were extracted automatically. Activity in all VOIs was calculated as the average radioactivity concentration (becquerels per cubic centimetre) in the left and right hemispheres. Radioactivity concentrations in the VOIs (kilobecquerels per cubic centimetre) or in parent compound-corrected arterial plasma (kilobecquerels per millilitre) were normalized in time–activity curves to the injected dose (ID) corrected for animal weight, in kilobecquerels per gram, thus yielding the unit grams per cubic centimetre and approximating the amount of uptake in terms of standardized uptake values (SUV).

Arterial input measurements were obtained for all PET tracers, except [^{11}C]Cimbi-88 for which full radiometabo-

lite information was not available, and distribution volumes (V_T) for VOIs were calculated based on the two tissue compartments (2TC) model using parent compound-corrected plasma input function as the arterial input function (Table 2). Assuming the specific 5-HT_{2A} receptor binding in the cerebellum is negligible [3], the non-displaceable binding potential (BP_{ND}) for all PET tracers were calculated applying the simplified reference tissue model (SRTM) [23]. Kinetic modelling was done in PMOD version 3.0 (PMOD Technologies).

HPLC analysis of pig plasma and brain tissue

Whole-blood samples (10 ml) drawn during PET scanning were centrifuged (1,500 $\times g$, 7 min at ambient temperature), and the plasma was filtered through a 0.45 μm filter (13 mm or 25 mm PVDF syringe filter; Whatman GD/X) before HPLC analysis with online radioactivity detection, as previously described [24].

Additionally, the presence of radioactive metabolites of [^{11}C]Cimbi-36 in the brain was investigated in two pigs. The pigs were killed by *i.v.* injection of pentobarbital 25 and 60 min after *i.v.* injection of about 500 MBq [^{11}C]Cimbi-36, and the brains were removed. Within 30 min of pentobarbital injection, brain tissue was homogenized in 0.1 N perchloric acid (Bie and Bentsen) saturated with sodium-EDTA (Sigma) for 2 $\times$ 30 s using a Polytron homogenizer. After centrifugation (1,500 $\times g$, 7 min at ambient temperature), the supernatant was neutralized using phosphate buffer, filtered (0.45 μm), and analysed by HPLC as described above. A plasma sample from blood taken at the time of decapitation was also analysed.

Statistical analysis

All statistical tests were performed using Prism version 5.0 (GraphPad software). *P* values below 0.05 were considered statistically significant. Results are expressed in means $\pm$ standard deviation (SD) unless otherwise stated.

Results

In vitro binding characterization

Affinities of the test compounds towards the 5-HT_{2A} receptor were measured against 0.2 nM [^3H]MDL100907 on GF62 cells stably transfected with the rat 5-HT_{2A} receptor, and the K_i values of the test compounds are given in Table 1. All tested compounds showed nanomolar affinity towards the 5-HT_{2A} receptor. Of the tested compounds, Cimbi-31 and Cimbi-138 showed the highest affinities for the 5-HT_{2A} receptor, and Cimbi-21 and Cimbi-88 showed the lowest

Table 1 In vitro 5-HT_{2A} receptor binding affinities and activation of PET tracer compounds

Compound	5-HT _{2A} antagonist binding ^a	5-HT _{2A} activation ^b	Intrinsic activity (%) ^c
Cimbi-5	1.49±0.35	1.02±0.08	91
Cimbi-21	12.5±3.11	50.7±12.3	86
Cimbi-27	1.12±0.08	0.19±0.03	99
Cimbi-29	1.36±0.37	29.7±2.82	93
Cimbi-31	0.16±0.04	1.06±0.19	83
Cimbi-36	1.01±0.17	0.51±0.19	87
Cimbi-82	2.89±1.05	2.31±0.11	88
Cimbi-88	47.2±16.3	6.39±0.97	84
Cimbi-138	0.35±0.05	0.96±0.18	92

^a K_i (nM ± SEM) measured against [³H]MDL100907 at GF-62 cells overexpressing rat 5-HT_{2A} receptors.

^b ED₅₀ values (nM ± SEM) for 5-HT_{2A} activation at GF-62 cells.

^c Mean maximal activation by test compound compared to 10 μM 5-HT.

affinities. PDSP screening results were obtained to determine whether the compounds had significant affinities for other neuroreceptors. According to the PDSP data, the K_i for Cimbi-36 against human 5-HT_{2A} receptors was 0.5±0.1 nM. Thus, Cimbi-36 was threefold more selective over 5-HT_{2C} (K_i 1.7±0.1 nM). At other targets tested by PDSP, Cimbi-36 was at least 120-fold more selective for 5-HT_{2A} receptors. The third highest affinity of Cimbi-36 was at Sigma 2 receptors (K_i 62 nM). The full PDSP screening results are given in Table S1 in the Supplementary material.

In vitro functional characterization

The functional properties of the compounds at the 5-HT_{2A} receptor were assessed by measuring their effect on PI

hydrolysis in GF62 cells overexpressing the 5-HT_{2A} receptor. All investigated compounds were found to be highly potent agonists at the 5-HT_{2A} receptor with EC₅₀ values in the nanomolar range (0.19–50.7 nM). For compounds previously tested, EC₅₀ values are in agreement with reported data [10]. For all compounds, pretreatment with 1 μM ketanserin completely inhibited the induced PI hydrolysis (data not shown). Furthermore, the degree of 5-HT_{2A} receptor activation achieved by the compounds was compared to the maximum effect of 5-HT (10 μM) in the same assays and reported as percentage of intrinsic activation. All compounds acted as full or nearly full agonists at the 5-HT_{2A} receptor, giving rise to 83–99% of the activation evoked by 10 μM 5-HT. The full results of in vitro activation are given in Table 1.

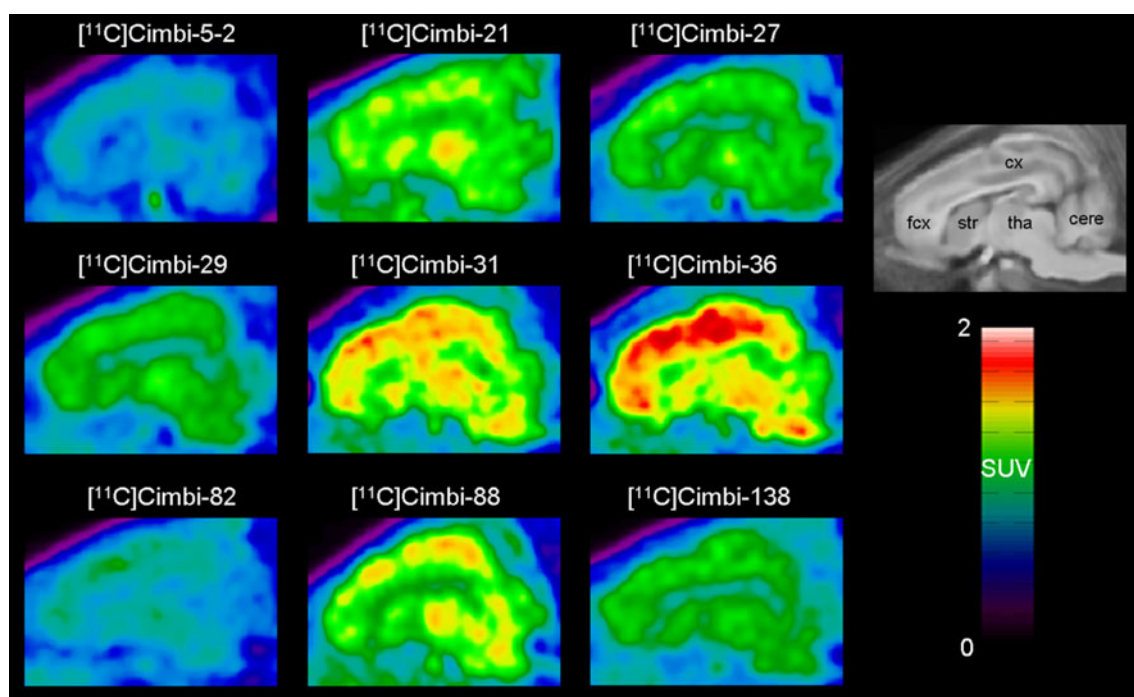


Fig. 2 Colour-coded sagittal PET images showing the average distribution of radioactivity in the pig brain from 10 to 90 min after i.v. injection of tracers. Right insert shows the corresponding sagittal

view of the MRI-based average atlas of the pig brain with structures labelled: fcx frontal cortex, cx cerebral cortex, tha thalamus, cere cerebellum, str striatum

In vivo biodistribution in pig brain

All ^{11}C -phenethylamines showed significant uptake in the pig brain as demonstrated in Fig. 2, and for all PET tracers, time–activity curves showed higher uptake in the cortex than in the cerebellum (Fig. 3). The peak cortical uptake varied among the tracers: [^{11}C]Cimbi-36 and [^{11}C]Cimbi-31 showed the highest uptake with a peak around 2.2 SUV, while [^{11}C]Cimbi-5-2 and [^{11}C]Cimbi-82 showed an uptake of only around 0.8 SUV and 1.2 SUV. The cortex-to-cerebellum uptake ratios, as measured by cortical SRTM BP_{ND} , are given in Table 2. Of the nine tested PET tracers, [^{11}C]Cimbi-21 and [^{11}C]Cimbi-88 showed the lowest cortex-to-cerebellum ratios with a cortical SRTM BP_{ND} value of 0.17. PET scanning with [^{11}C]Cimbi-5-2, [^{11}C]Cimbi-27, [^{11}C]Cimbi-31 and [^{11}C]Cimbi-82 gave cortical

SRTM BP_{ND} values similar to that found with [^{11}C]Cimbi-5 (0.46 $\pm$ 0.11). [^{11}C]Cimbi-36, and [^{11}C]Cimbi-138 showed the highest cortical-to-cerebellum uptake ratios with cortical SRTM BP_{ND} values of 0.60 and 0.82, indicative of high target-to-background ratios with these tracers. For all regional time–activity curves, the peak radioactivity concentration occurred 10–20 min after injection and thereafter declined, implying that binding was reversible over the 90-min scan time. The time–activity curves showed that regional activity of [^{11}C]Cimbi-5-2 and [^{11}C]Cimbi-88 in the pig brain declined at a slower rate than that of the other PET tracers. [^{11}C]Cimbi-21, [^{11}C]Cimbi-31, [^{11}C]Cimbi-36 and [^{11}C]Cimbi-82 showed a more rapid decline in regional brain radioactivity indicating faster kinetics with these tracers. V_T was calculated using the 2TC model with metabolite-corrected arterial plasma radioactivity as input function

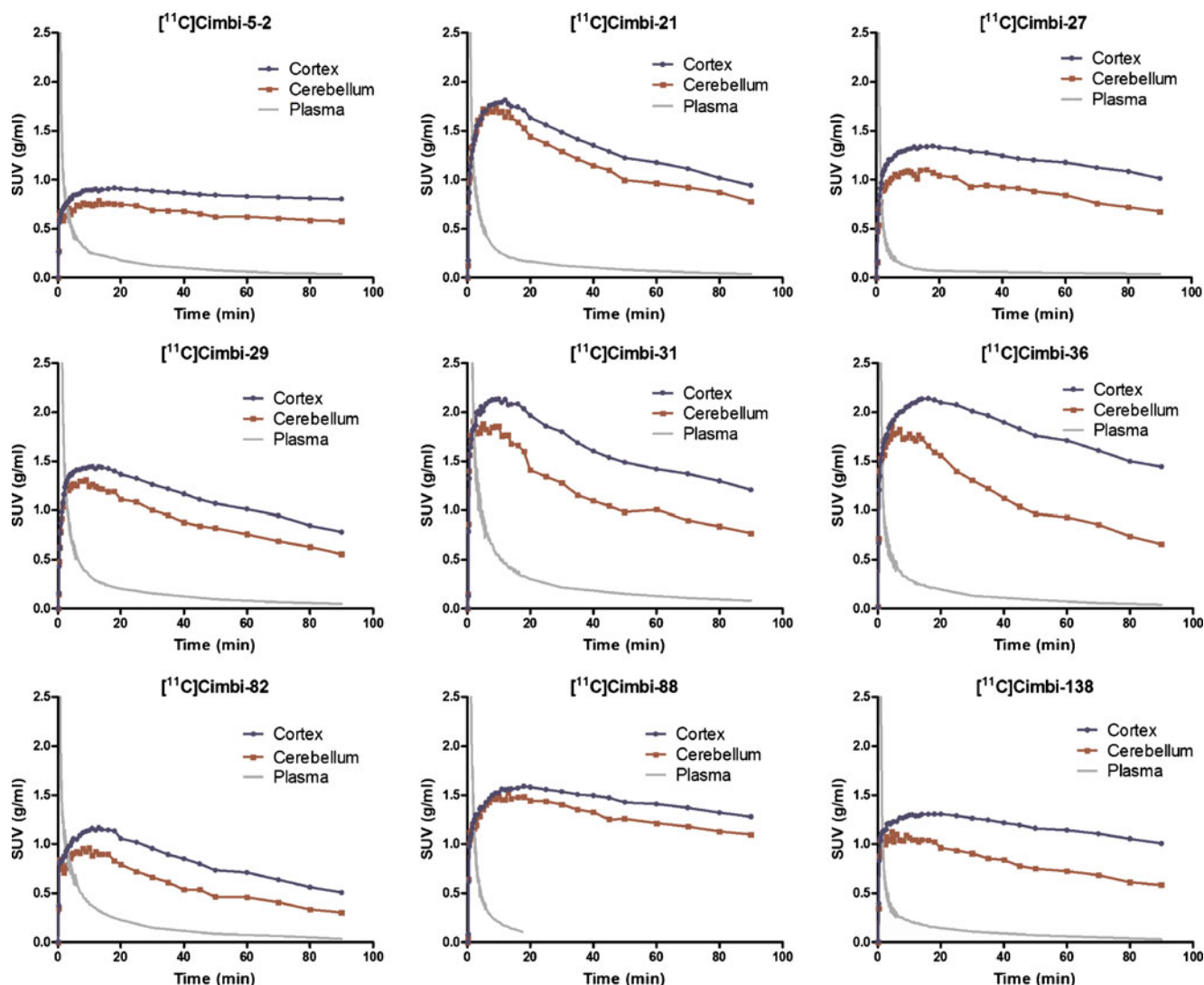


Fig. 3 Regional time–activity curves of ^{11}C -phenethylamines in the pig brain (blue circles cortex, red squares cerebellum, grey solid lines parent compound-corrected plasma). Standardized uptake values (SUV) in pig brain are shown for each tracer

Table 2 PET tracers in pig brain: injection data, cLogD values, free fraction and in vivo biodistribution as calculated by kinetic modelling

Tracer	Injected dose (MBq) ^a	Specific activity (GBq/ μ mol) ^a	cLogD ^b	Plasma free fraction (%)	2TC distribution volumes		SRTM Cortical BP _{ND}
					Cortex	Cerebellum	
[¹¹ C]Cimbi-5-2	682	122.7	3.33	0.4	10.93	6.88	0.32
[¹¹ C]Cimbi-21	627	9.6	3.86	0.8	7.24	5.73	0.17
[¹¹ C]Cimbi-27	434	21.6	2.94	0.7	16.79	11.00	0.45
[¹¹ C]Cimbi-29	710	45.2	3.35	0.4	5.15	3.57	0.32
[¹¹ C]Cimbi-31	390	36.3	2.87	1.0	dnf	dnf	0.43
[¹¹ C]Cimbi-36	506	210.4	3.42	1.1	13.42	6.76	0.82
[¹¹ C]Cimbi-82	578	445.5	3.49	0.7	4.51	2.82	0.49
[¹¹ C]Cimbi-88	462	231.2	-0.24	6.5	n.d.	n.d.	0.17
[¹¹ C]Cimbi-138	589	455.6	4.40	1.2	13.85	6.19	0.60

dnf did not fit kinetic model, n.d. not determined.

^a At time of injection.

^b Calculated using CSLogD (ChemSilico).

(Table 2). Due to missing radiometabolite data, 2TC V_T could not be calculated for [¹¹C]Cimbi-82, while the [¹¹C]Cimbi-31 data did not fit the 2TC model.

Ketanserin blockade of [¹¹C]Cimbi-36 in vivo

In a single pig, the effect of pretreatment with ketanserin on [¹¹C]Cimbi-36 binding was examined. In this baseline scan, the cortical SRTM BP_{ND} of [¹¹C]Cimbi-36 was 0.70. Following i.v. administration of 10 mg/kg ketanserin 30 min prior to a second scan, BP_{ND} was decreased to 0.26. Also, the time–activity curves indicated that pretreatment with ketanserin decreased cortical [¹¹C]Cimbi-36 binding (Fig. 5). However, the ketanserin blockade was not complete as indicated by the persistent difference between the cortical and cerebellar radioactivity concentrations in the blocked time–activity curves (Fig. 5).

Radiolabelled metabolites

For all compounds with full metabolite data, the relative amount of parent compound in plasma declined exponentially at similar rates, and 5–9% remained in plasma 90 min after injection (see Fig. 6 for an example). In the radio-HPLC chromatograms of pig plasma taken 30 min after i.v. injection of the PET tracers, a distinct peak was seen for most of the PET tracers eluting prior to the parent compound (Fig. 4). This lipophilic radiolabelled metabolite reached a maximum in plasma at around 20–40 min after injection and then dropped off slightly up to 90 min (see Fig. 6 for an example). In the HPLC analysis of homogenized pig brain tissue taken 20 min after [¹¹C]Cimbi-36 injection, only negligible amounts of this metab-

olite were found in frontal cortex tissue compared to plasma obtained at the same time (Fig. 7). The brain tissue obtained 60 min after i.v. injection of [¹¹C]Cimbi-36 contained insufficient radioactivity for reliable HPLC analysis.

Discussion

We present here the radiosynthesis and biological evaluation of a series of substituted phenethylamines as 5-HT_{2A} receptor agonist PET tracers. Based on an in vivo screening approach involving a HRRT PET scan with each tracer, we identified [¹¹C]Cimbi-36 as the most promising candidate and conducted further studies with this compound. [¹¹C]Cimbi-36 had a higher brain uptake and improved target-to-background binding ratio over the previously validated candidate [¹¹C]Cimbi-5 (cortical SRTM BP_{ND} 0.46±0.11) [12]. We used SRTM BP_{ND} to evaluate the target-to-background binding ratios of the PET tracers since cerebellum generally is a valid reference region for quantification of 5-HT_{2A} receptor binding [3, 25]. Time–activity curves from [¹¹C]Cimbi-36 showed the highest brain uptake (peak cortical SUV 2.2), and the greatest separation between cortical and cerebellar uptake (cortical SRTM BP_{ND} 0.82) of the nine tested compounds. Thus, the target-to-background ratio of [¹¹C]Cimbi-36 was higher than those of both [¹¹C]Cimbi-5 and the eight other tested PET tracers. Although [¹¹C]Cimbi-138 also displayed promising PET tracer properties with a SRTM BP_{ND} of 0.60 and a peak cortical uptake of 1.4, [¹¹C]Cimbi-36 was superior to [¹¹C]Cimbi-138 in both measures. 5-HT_{2A} receptor blocking with ketanserin resulted in a reduction in the cortical SRTM BP_{ND} from 0.70 to 0.26, supporting the view that [¹¹C]Cimbi-36

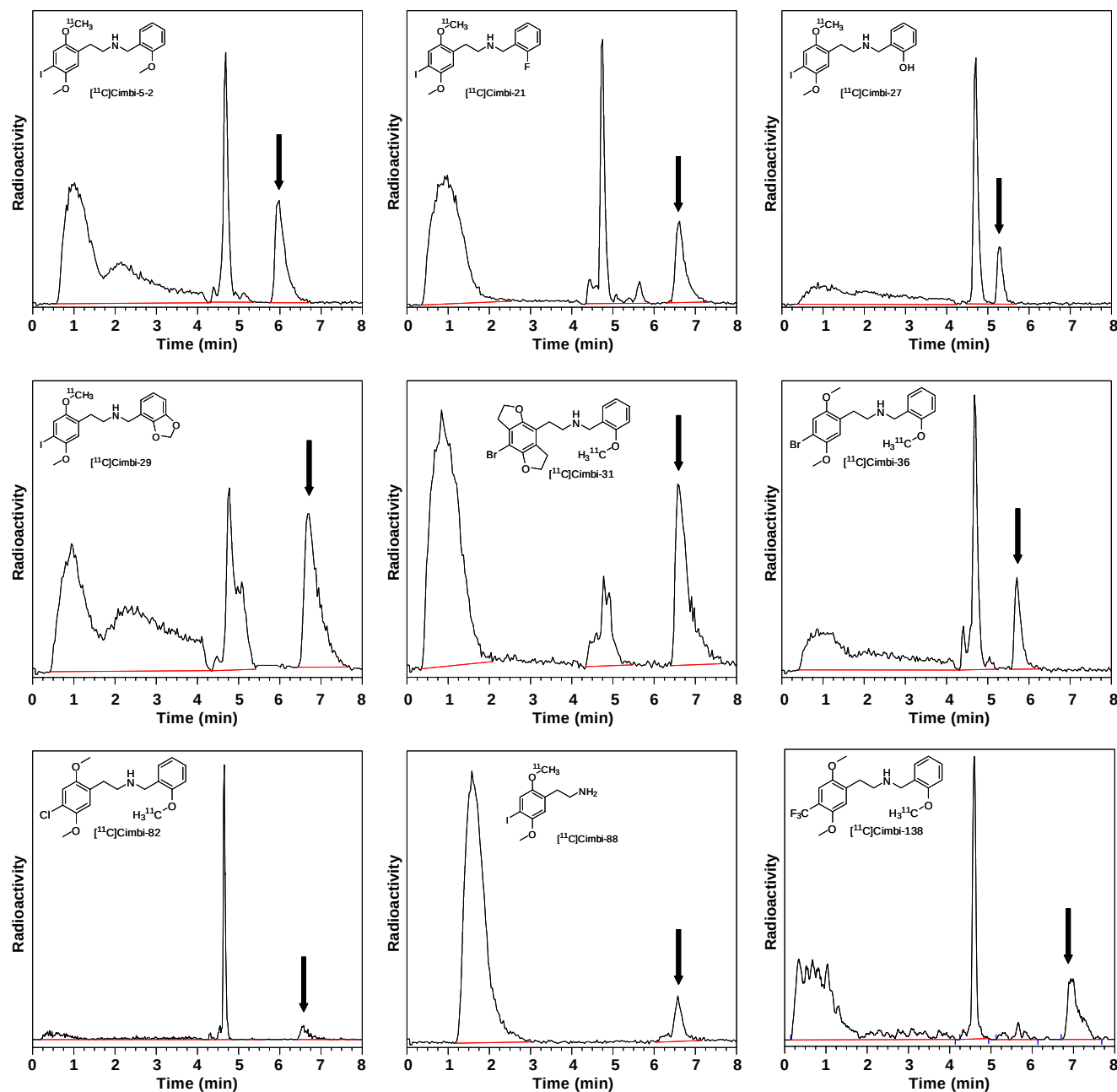


Fig. 4 HPLC radiochromatograms of pig plasma 30 min after injection (10 min for [^{11}C]Cimbi-88); *black arrows* indicate parent compounds. Eluent compositions were adjusted so that each parent compound eluted at 5–7 min retention time

binding in the pig cortex represents 5-HT_{2A} receptor binding. Furthermore, the PDSP screening results confirmed that Cimbi-36 was highly selective over non-5-HT₂ targets. Although Cimbi-36 was only threefold selective for 5-HT_{2A} receptors over 5-HT_{2C} receptors, cortical [^{11}C] Cimbi-36 signal could be attributed to 5-HT_{2A} receptor binding since the density 5-HT_{2C} receptors is negligible compared to the density of 5-HT_{2A} receptors in the cortical areas [26, 27].

PET tracers such as [^{11}C]Cimbi-21 and [^{11}C]Cimbi-88 were discarded for further studies based on their low target-to-background binding ratio in the screening procedure. The cortical SRTM BP_{ND} of these PET tracers were lower in the pig brain than that of the previously validated candidate [^{11}C]Cimbi-5. It should be noted that the [^{11}C] Cimbi-21 scan was conducted with lower specific radioactivity than the scans with the other compounds and [^{11}C] Cimbi-21 target-to-background binding ratio may have

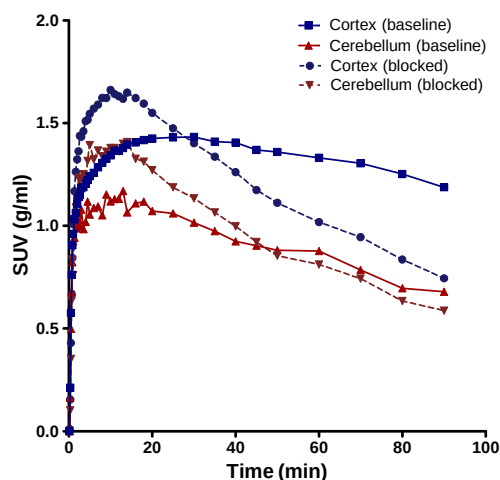


Fig. 5 Cortical and cerebellar time-activity curves of [^{11}C]Cimbi-36 from a pig brain at baseline (blue) and following pretreatment with 10 mg/mg ketanserin (red). The SUVs are normalized to injected dose per body weight. Cortical SRTM BP_{ND} of [^{11}C]Cimbi-36 was 0.70 at baseline and 0.26 after ketanserin pretreatment

been lower due to this, but it is unlikely that improving the specific radioactivity in a repeat scan with this tracer would have improved cortical BP_{ND} to the level of [^{11}C]Cimbi-36. [^{11}C]Cimbi-5-2, [^{11}C]Cimbi-27, [^{11}C]Cimbi-29, [^{11}C]Cimbi-31 and [^{11}C]Cimbi-82 showed similar brain uptake and cortical SRTM BP_{ND} as the previously labelled candidate PET tracer. Given the close structural resemblance between these compounds, it is perhaps not surprising that they showed similar properties in vivo in the pig brain.

The regional time-activity curves for [^{11}C]Cimbi-36 clearly declined over the 90 min scanning time meaning that [^{11}C]Cimbi-36 shows reversible binding to 5-HT $_{2A}$ receptor during PET scanning. The time-activity curves of [^{11}C]Cimbi-36 also seemed more reversible than, for example, [^{11}C]Cimbi-5-2, [^{11}C]Cimbi-27 and [^{11}C]Cimbi-138. Reversible binding kinetics is advantageous for quantification [28], and the faster kinetics of [^{11}C]Cimbi-36 may prove important when moving into clinical studies in humans where kinetics are usually slower.

The in vitro binding results confirmed that all compounds tested as PET tracers had high affinity for the 5-HT $_{2A}$ receptor and that all compounds, as expected based on their phenethylamine structure, activated 5-HT $_{2A}$ receptors with EC_{50} values in the nanomolar range, and thus are indeed 5-HT $_{2A}$ receptor agonists. This is in agreement with previous data showing that Cimbi-5, Cimbi-27, Cimbi-29 and Cimbi-36 are selective and high-affinity agonists [10]. Cimbi-21 and Cimbi-88 had the lowest 5-HT $_{2A}$ receptor affinity and lower EC_{50} values than most of the other tested compounds, and they also showed the lowest target-to-background binding ratios in the in vivo studies. Since the binding potential of a PET tracer is proportional to its affinity [28], it is not surprising that the compounds with the lowest affinity also gave the lowest

cortical SRTM BP_{ND} . However, Cimbi-31 showed the highest 5-HT $_{2A}$ receptor affinity, and in this respect, it is perhaps somewhat surprising that [^{11}C]Cimbi-31 did not seem to bind receptors more irreversibly as compared to some of the other tracers as indicated by rate of washout from the cortical region. However, this testifies to the complexity of the binding in the living brain as compared to affinity constants measured in vitro. The in vivo properties of a PET tracer are influenced by several factors, including brain uptake and transport, binding kinetics and very prominently non-specific binding. We report here roughly similar in vitro binding and activation properties of Cimbi-36, Cimbi-5, Cimbi-27, Cimbi-29 and Cimbi-82, yet [^{11}C]Cimbi-36 was a markedly better PET tracer with higher target-to-background ratios compared to all these compounds.

Thus, in this series of substituted ^{11}C -phenethylamines we demonstrated that minor structural changes may alter the PET tracer properties of a compound without greatly changing its in vitro properties. With these nine tested PET tracers there was no apparent relationship between calculated LogD values and nonspecific uptake as determined by cerebellum V_{T} . This indicates that the nonspecific binding for phenethylamine PET tracers is dependent on factors other than just lipophilicity, and that lipophilicity alone is not a solid predictor of the level of nonspecific binding of a PET tracer in vivo, which has also been suggested previously [29].

Most of the tested *N*-benzyl substituted ^{11}C -phenethylamines gave rise to a distinct lipophilic radiolabelled metabolite. We were unable to determine the identity of these labelled metabolites in pig plasma based on the retention time on the radiochromatograms alone. It is proposed that they result from an *O*-demethylation at the 2- or 5-methoxy position in the phenethylamine moiety. Several lines of evidence support this speculation. Firstly, DOI has been shown to be metabolized through demethylation at either or both methoxy groups [30]. Further, the radiochromatograms for [^{11}C]Cimbi-31 (which has no methoxy groups in the

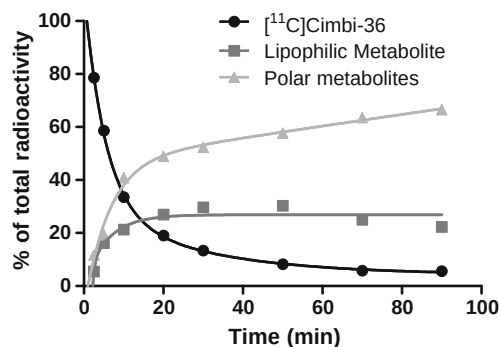


Fig. 6 HPLC analysis of radioactive metabolites in pig plasma after i.v. injection of [^{11}C]Cimbi-36. The amounts of parent compound (circles), lipophilic metabolite (squares) and polar metabolites (triangles) are shown as percent of total radioactivity

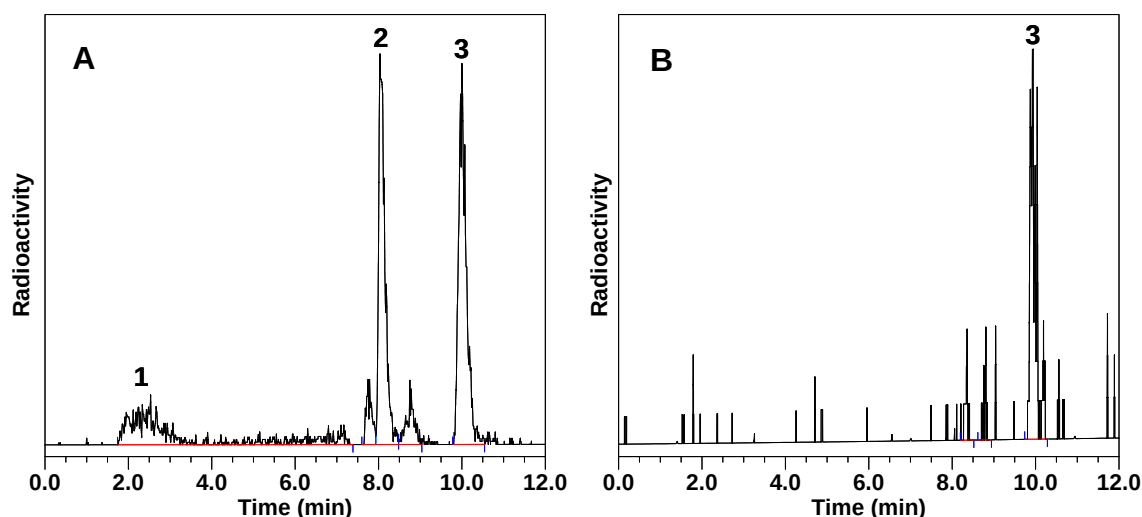


Fig. 7 HPLC analysis of plasma (a) and frontal cortex extract (b) 20 min after injection of [^{11}C]Cimbi-36. Peaks: 1 polar metabolites, 2 lipophilic metabolites, 3 parent compound

phenethylamine moiety) only showed small amounts of lipophilic metabolites. Also, the demethylation products of [^{11}C]Cimbi-88 would be rather polar and therefore would be expected to elute with polar metabolites, as was the case. The metabolism of Cimbi-88 has also been reported to involve demethylation at both the 2- and 5-methoxy positions [31]. Changing the 4-substituent from iodine to bromine, chlorine or trifluoromethyl in the phenethylamine moiety had a considerable effect on the amount of radiolabelled metabolite in plasma. Since the parent compound in plasma declined similarly over time for all compounds ([^{11}C]Cimbi-5-2, [^{11}C]Cimbi-36, [^{11}C]Cimbi-82 and [^{11}C]Cimbi-138), this difference is probably caused by variation in the rate of further metabolism of the lipophilic metabolites. However, further studies would be needed to uniquely identify the *in vivo* metabolic route of these substituted phenethylamines.

Given that a radiolabelled metabolite of [^{11}C]Cimbi-36 was present in pig plasma, we investigated the possible presence of the metabolite in pig brain. Although a substantial amount of radiometabolite was present in plasma 20 min after *i.v.* injection of [^{11}C]Cimbi-36, the radiometabolite was barely detectable in frontal cortex tissue from the same pig (Fig. 7). This suggests that the radiometabolite of [^{11}C]Cimbi-36 does not enter the brain to any extent that would interfere with the [^{11}C]Cimbi-36 signal or decrease the binding potential *in vivo*. Based on our data, however, we cannot firmly dismiss the presence of some radiometabolites in pig brain since some small peaks were observed in the radiochromatograms which concurrently were noisy due to the dilution of the tissue needed for homogenization. In contrast, plasma is loaded directly to the HPLC without dilution, and thus the chromatogram was less noisy. Although on the basis of the present results we cannot rule out the presence of radiolabelled metabolites in pig brain, a much lower fraction was

clearly present in brain compared to plasma, and the small amounts in brain implied by the tissue radiochromatogram may have been derived from blood present in the vascular compartments of the brain tissue.

All the *N*-benzyl-substituted ^{11}C -phenethylamines had a low free fraction in pig plasma (0.4–1.5%); a low free plasma fraction should theoretically impair brain uptake. However, since many of these compounds with similar free fractions showed different degrees of brain uptake as measured by peak SUV, it seems that the level of brain uptake is not markedly influenced by the fraction of free tracer in plasma. The only non-*N*-benzyl-substituted compound, [^{11}C]Cimbi-88, had a higher free fraction in pig plasma (6.5%) which is in accordance with this compound being less lipophilic than the *N*-benzyl-substituted tracers. However, brain uptake of [^{11}C]Cimbi-88 was lower than that of [^{11}C]Cimbi-36, [^{11}C]Cimbi-27 and [^{11}C]Cimbi-31.

Two PET scans were performed with [^{11}C]Cimbi-36 and [^{11}C]Cimbi-82 at relatively low specific radioactivities (6.9 and 9.0 GBq/ μmol , respectively; data not shown). In these scans, lower cortical BP_{ND} were found for both tracers, suggesting that tracer doses for 5-HT $_2\text{A}$ receptor agonist binding were exceeded. Thus, future PET studies with [^{11}C]Cimbi-36 should be conducted with an injected mass of <10 μg cold dose.

Conclusion

Our *in vivo* screening of phenethylamine 5-HT $_2\text{A}$ receptor agonist PET tracers led to the identification of [^{11}C]Cimbi-36 as the most promising PET tracer for further investigations. [^{11}C]Cimbi-36 showed the highest target-to-background

binding ratio of all compounds tested, and it was also displaceable by ketanserin in vivo, supporting its 5-HT_{2A} receptor selectivity. In vitro studies confirmed that [¹¹C]Cimbi-36 is a highly potent, high-affinity and selective 5-HT_{2A} receptor agonist. Thus, [¹¹C]Cimbi-36 is currently the most promising PET tracer for imaging cerebral 5-HT_{2A} receptor agonist binding in the living brain.

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Conflicts of interest None.

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