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Letter

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Discovery of a Novel Muscarinic Receptor PET Radioligand with Rapid Kinetics in the Monkey Brain

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Abstract

Positron emission tomography (PET), together with a suitable radioligand, is one of the more prominent methods for measuring changes in synaptic neurotransmitter concentrations *in vivo*. The radioligand of choice for such measurements on the cholinergic system has been the muscarinic receptor antagonist *N*-[1-^{11}C]propyl-3-piperidyl benzilate (PPB). In an effort to overcome the shortcomings with the technically cumbersome synthesis of [^{11}C]PPB, we designed and synthesized four structurally related analogs of PPB, of which (*S,R*)-1-methylpiperidin-3-yl)2-cyclopentyl-2-hydroxy-2-phenylacetate (**1**) was found to bind muscarinic receptors with similar affinity as PPB (3.5 vs. 7.9 nM, respectively). (*S,R*)-**1** was radiolabeled via *N*-^{11}C-methylation at high radiochemical purity (>99%) and high specific radioactivity (>130 GBq/μmol). *In vitro* studies by autoradiography on human brain tissue and *in vivo* studies by PET in non-human primates demonstrated excellent signal to noise ratios and a kinetic profile in brain comparable to that of [^{11}C]PBB. (*S,R*)-[^{11}C]**1** is a promising candidate for measuring changes in endogenous acetylcholine concentrations.

Keywords

PET, radioligand, acetylcholine, muscarinic, receptor, carbon-11

Positron emission tomography (PET) is a non-invasive imaging technique increasingly used in translational biomedical research and drug development. A key feature of PET is that it facilitates investigations of endogenous synaptic neurotransmitter concentrations in the living brain.[1, 2] This can provide critical understanding of the pathophysiology of neuropsychiatric and neurodegenerative disease, while aiding the development of new central nervous system drugs that aim to increase synaptic neurotransmitter concentrations. Although this experimental paradigm has been successfully applied for several receptor systems,[3] there is a general lack of radioligands that are sensitive to changes in endogenous neurotransmitter concentration.

Muscarinic acetylcholine (mACh) receptors were imaged in the human brain over three decades ago using *N*-[^{11}C]methyl benztropine.[4] Since then, a number of PET radioligands for mACh receptors have been reported, including [^{11}C]scopolamine,[5] [^{11}C]quinuclidinyl-benzylate ([^{11}C]-QNB),[6] *N*-[^{11}C]methylpiperidyl-benzylate ([^{11}C]MPB)[7] and *N*-[^{11}C]propyl-3-piperidyl benzilate ([^{11}C]PPB) (Figure 1),[8] among which only a few have demonstrated sensitivity towards endogenous ACh concentrations[8]. One of the frontrunner radioligands for this purpose is [^{11}C]PPB which was identified from a homologous series of *N*-alkyl analogs that also included its *N*-methyl and *N*-ethyl analogs, [^{11}C]MPB and [^{11}C]EPB.[8] Interestingly, in this series of molecules, the sensitivity towards endogenous ACh was inversely correlated to binding affinity and the least potent analog, [^{11}C]PPB, was found to be most sensitive to endogenous neurotransmitter concentrations. A drawback with [^{11}C]PPB is that its synthesis proceeds via *N*-^{11}C-propylation; this is technically cumbersome and thus hampers widespread use of the radioligand. The aim of this work was to develop an equipotent analog of PBB that could be labeled using the most commonly used ^{11}C-labeled precursor, ^{11}C-methyl iodide, and evaluate it as a candidate radioligand for mACh receptors *in vitro* and *in vivo*.

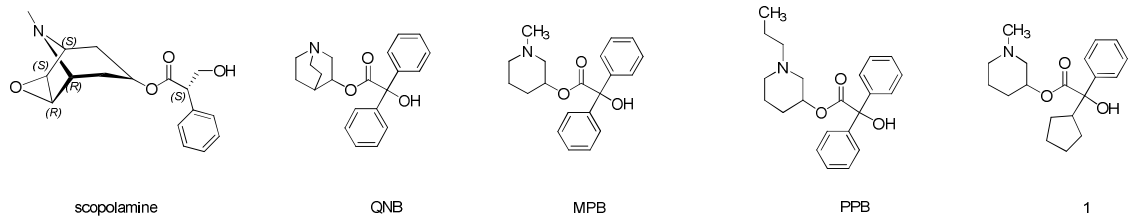
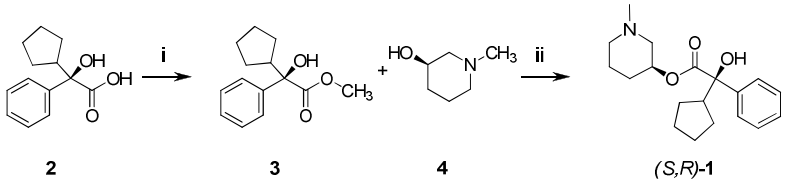


Figure 1. Chemical structures of muscarinic receptor antagonists used in PET imaging.

Our lead molecule in this study, PBB, was reported along with a series of psychotomimetic glycolate esters by Baumgold et al. in 1975.[9] Evaluation of the described structure-activity relationship on this scaffold indicated that substitution of one of the phenyls with a cyclopentyl group resulted in a reduction in affinity towards mACh.[9] Although only racemic mixtures were evaluated in the original publication, the substitution of a phenyl with a cyclopentyl group was considered attractive since it would introduce an additional stereocenter. The chemistry would thus provide four stereomers that could be evaluated against PBB for muscarinic receptor *in vitro* affinity. Thus, a chemical route was designed that provided the four stereomers of **1** in two steps from phenylacetic acid **2**, via esterification and chiral separation using supercritical fluid chromatography (SFC) (Scheme 1).



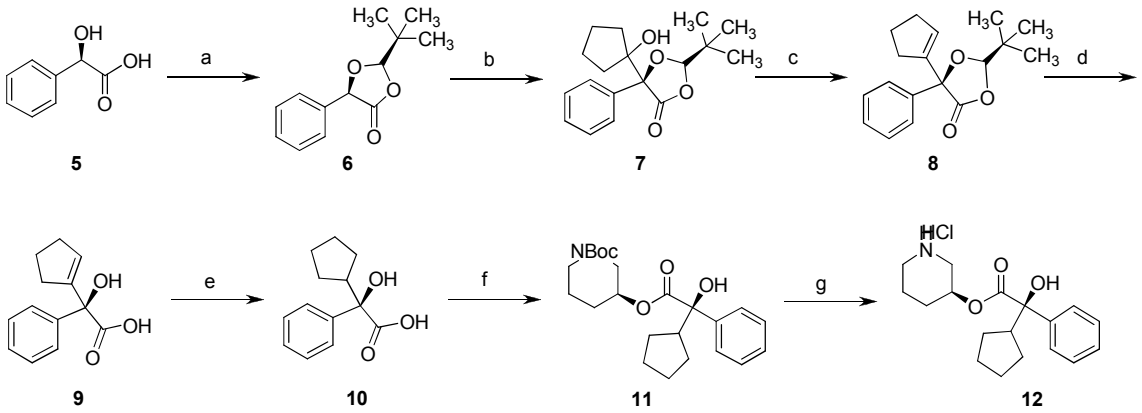
Scheme 1. Synthesis of the four stereomers of **1**, here exemplified with the preparation of (*S,R*)-**1**; i) methanol, sulfuric acid, reflux then SFC on ChiralCel OD-H with 95:5% heptane / *i*-propanol with 0.1% dimethyl-ethylamine; ii) sodium hydride, heptane, reflux.

A high affinity assay for human muscarinic 1 receptor (hM1) was performed to identify a potential candidate radioligand from the newly synthesized compounds. Gratifyingly, three stereomers of **1** showed similar or higher potency as compared to (+)-PPB (see Table 1) and were selected for further evaluation *in vivo*. The affinity of the (S,S)-isomer was deemed to low to enable imaging of mAChR in vivo.

Table 1. Results from a high affinity assay for the human muscarinic 1 receptor (hM1)

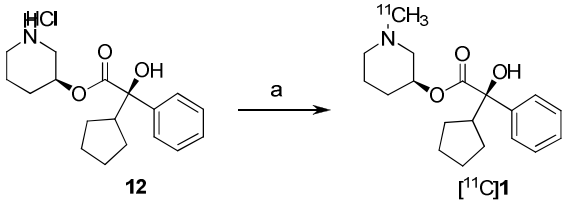
Compound	K_i **(nM)**
(*S,S*)**-1**	73.6
(*R,S*)**-1**	13.4
(+)-PPB	7.9
(*S,R*)**-1**	3.5
(*R,R*)**-1**	0.5

Next, a stereoselective route was designed to provide the desired optically pure *N*-desmethyl precursor materials for the three most potent stereomers of **1**. Starting from phenyl hydroxy acid **5**, dioxolanone **7** was obtained via stereospecific alkylation of intermediate **6** (>99.9% ee). Dehydration, saponification and catalytic hydrogenation furnished compound **10**, which was converted into **11** by reaction with (*S*)-1-Boc-3-hydroxypiperidine under Mitsunobu conditions. This reaction proceeded with a modest yield of 50% and hence was the lowest yielding reaction in the synthetic sequence. Acidic deprotection finally yielded the target molecule in >99.9% diastereomeric excess and in a 20% overall yield from **5**.



Scheme 2. Synthesis of the *N*-desmethyl precursor to **1**, here exemplified by the (*S,R*)-isomer. Reagents and conditions: (a) pivalaldehyde, cat. TfOH, pentane, reflux (85%); (b) cyclopentanone, LHMDS, THF, -78°C, >99.9% de (65%); (c) $SOCl_2$, pyridine, THF, 0°C (99%); (d) (i) KOH, MeOH, reflux, (ii) HCl (100%); (e) H_2, Pd/C, MeOH, rt, >99.9% ee (80%); (f) (*S*)-1-Boc-3-hydroxypiperidine, DIAD, PPh_3, THF, 25°C (50%); (g) (i) TFA, CH_2Cl_2, rt, (ii) HCl, >99.9% de (93%).

The three more potent stereomers of [^{11}C]**1** ((*S,R*), (*R,S*) and (*R,R*)) were synthesized by *N*-[^{11}C]methylation of their corresponding des-methyl precursors (**12**) with [^{11}C]methyl triflate (Scheme 1). The radiochemical conversion of [^{11}C]methyl triflate into [^{11}C]**1** was greater than 80% and the total time of synthesis was approximately 32 min, producing on average 3 GBq of (*S,R*)-[^{11}C]**1,** (*R,S*)-[^{11}C]**1**, and (*R,R*)-[^{11}C]**1** at high specific radioactivity (130-230 GBq/μmol). The products were formulated in a solution of ethanol (5%) in phosphate buffered saline (pH 7.4) for subsequent PET imaging experiments in non-human primates. The formulations of [^{11}C]**1** were radiochemically pure (>99%) and stable for at least 60 min after completed synthesis.



Scheme 3. Radiolabeling of [^{11}C]**1**, here exemplified by the (*S,R*)-isomer. Reagents and conditions: a) [^{11}C]MeOTf, acetone, NaOH, rt

The three [^{11}C]**1** stereomers were evaluated by subsequent PET measurements in the same cynomolgus monkey on the same experimental day. Following intravenous administration of the individual radiolabeled stereomers, radioactivity readily entered the brain and distributed heterogeneously in tissue (Fig 3). One major radiometabolite was observed in monkey plasma that was more polar than the parent compound. For all three stereomers the concentration of radioactivity was higher in mACh receptor rich regions (cortex, striatum) than in cerebellum, a region known to be almost devoid of mACh receptors.[10, 11] The ratio of radioactivity between target regions and cerebellum was proportional to the binding affinity of the individual stereomers. Thus, the greatest radioactivity ratio was observed for (*R,R*)-[^{11}C]**1** and the lowest ratio was observed for (*R,S*)-[^{11}C]**1**. Based on the work by Tsukada et al.,[8] it was hypothesized that the binding kinetics of the radioligand were important for sensitivity towards endogenous ACh. Time-activity curves (TACs) for the regional distribution of radioactivity following administration of the three stereomers were generated and compared. The shape of the TACs for target regions was found to be related to binding affinity, with the highest affinity stereomer, (*R,R*)-[^{11}C]**1** showing near absence of wash-out from target regions during the time-frame of the PET measurement (123 min). Gratifyingly, however, the TACs for (*S,R*)-[^{11}C]**1** showed a rapid decrease in target regions as well as high contrast between target and non-target regions. Thus, (*S,R*)-[^{11}C]**1** showed a similar kinetic profile in brain as

that reported for [^{11}C]PBB and was therefore selected for further evaluation by *in vitro* and *in vivo* challenge studies.

A series of *in vitro* autoradiography studies were next performed to investigate (*S,R*)-[^{11}C]**1** binding to whole hemispheres from the post-mortem human brain. First, the binding was fully blocked with the non-selective mACh antagonist scopolamine, thus demonstrating binding specificity towards muscarinic receptors in the human brain. Second, the binding was found to be partially blocked by the M1 selective antagonist pirenzepine, indicating that there is a degree of M1 selectivity in the binding of (*S,R*)-[^{11}C]**1**. Third, the binding was found to be unaffected by co-incubation with the selective ACh esterase inhibitor, donepezil. The last observation was important to rule out any competition between donepezil and (*S,R*)-[^{11}C]**1** at mACh receptors. An effect of donepezil on the binding of (*S,R*)-[^{11}C]**1** *in vivo* might thus be attributed to the increase of synaptic ACh and would not be a result from a direct interaction of donepezil with a shared binding site in brain tissue.

Finally, the specific binding of (*S,R*)-[^{11}C]**1** in the non-human primate brain was investigated in a pre-treatment PET experiment. In this experiment, a baseline PET measurement with (*S,R*)-[^{11}C]**1** was followed by a pre-treatment PET measurement. In the latter experiment, the injection of (*S,R*)-[^{11}C]**1** was preceded by treatment with the mACh receptor antagonist scopolamine (0.05 mg/kg). Following scopolamine, the radioactivity in target regions was markedly reduced to the same level as cerebellum, thus confirming high level of binding specificity towards muscarinic receptors *in vivo* (Fig. 3 B,D).

Conclusions

(*S,R*)-[^{11}C]**1** has good characteristics for visualization of mACh receptors *in vitro* and *in vivo*. Further studies are required to elucidate its sensitivity towards endogenous ACh *in vivo*.

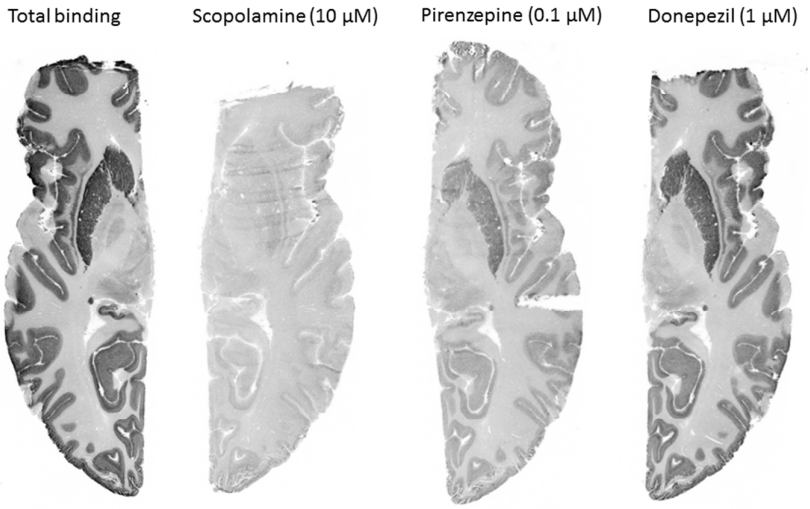


Figure 2. Autoradiograms of horizontal hemispheres from the post-mortem human brain incubated with (*S,R*)-[^{11}C]**1**.

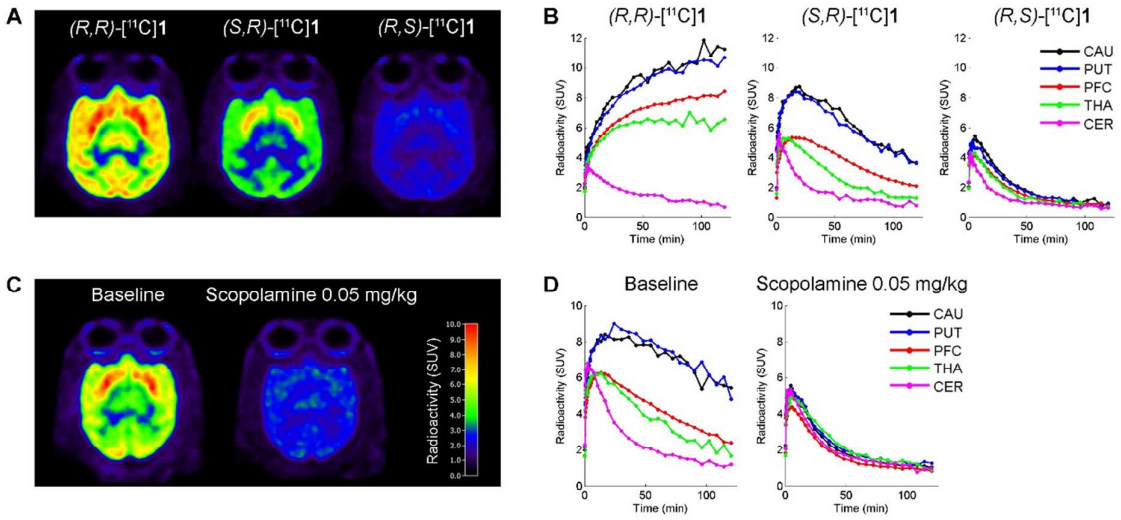


Figure 3. PET images and time-activity curves (TACs) following intravenous injection of [^{11}C]**1** in cynomolgus monkey. (A) Color coded PET images showing distribution of radioactivity in the monkey brain after radioligand injection. Summation images from 3 to 123 minutes are shown. Image intensity was normalized for injected radioactivity and body weight. (B) Representative TACs for brain regions of interest during baseline measurements. (C) Color coded PET images showing distribution of radioactivity in the monkey brain after injection of (*S*,*R*)-[^{11}C]**1** and after pretreatment with scopolamine. Summation images from 3 to 123 minutes are shown. Image intensity was normalized for injected radioactivity and body weight. (D) Time-activity curves for brain regions following pretreatment with scopolamine (0.05 mg/kg). CAU, Caudate; PUT, putamen; PFC, prefrontal cortex; THA, thalamus; CER, cerebellum.

Methods

Animals and study design

The study was approved by the Animal Ethics Committee of the Swedish Animal Welfare Agency (Dnr 452/11) and was performed according to the "Guidelines for planning, conducting and documenting experimental research" (Dnr 4820/06-600) of Karolinska Institutet, the "Guide for the Care and Use of Laboratory Animals",[12] the AstraZeneca Bioethical Policy and the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Two female cynomolgus monkeys (body weights, 5.6 and 7.1 kg) were examined in this study. The monkeys were owned by the Centre for Psychiatry Research, Department of Clinical Neuroscience, Karolinska Institutet, and housed in the Astrid Fagraeus Laboratory of the Swedish Institute for Infectious Disease Control, Solna, Sweden.

Five PET measurements were performed in two experimental sessions. In the first session, baseline PET measurements were performed after intravenous injection of (*S,R*)-[^{11}C]**1,** (*R,S*)-[^{11}C]**1**, and (*R,R*)-[^{11}C]**1** (202-212 MBq). In the second session, a baseline PET measurement was first performed with (*R,S*)-[^{11}C]**1** (208 MBq). This was followed by a pre-treatment PET experiment, in which the PET measurement with (*R,S*)-[^{11}C]**1** (208 MBq) was preceded by the intravenous infusion of a solution of scopolamine (0.05 mg/kg) over 10 minutes.

PET experimental procedure

Anaesthesia was induced by intramuscular injection of ketamine hydrochloride (approximately 10 mg/kg) and maintained by the administration of a mixture of sevoflurane (2-8%), oxygen and medical air after endotracheal intubation. The head was immobilized with

a fixation device.[13] Body temperature was maintained by a Bair Hugger model 505 (Arizant Healthcare, MN) and monitored by an esophageal thermometer. Heart rate, respiratory rate and oxygen saturation were continuously monitored throughout the experiments. Blood pressure was monitored every 15 min. Fluid balance was maintained by a continuous infusion of saline.

In each PET measurement, a sterile physiological phosphate buffered (pH 7.4) saline (PBS) solution containing radiolabeled drug, in a volume not exceeding 5 mL, was injected as a bolus into a sural vein during 5 s. PET data acquisition started at time of the bolus injection.

PET measurements

PET measurements were conducted using the High Resolution Research Tomograph (HRRT) (Siemens Molecular Imaging). List-mode data were reconstructed using the ordinary Poisson-3D-ordered subset expectation maximization (OP-3D-OSEM) algorithm, with 10 iterations and 16 subsets including modelling of the point spread function (PSF). The corresponding in-plane resolution with OP-3D-OSEM PSF was 1.5 mm fullwidth at half-maximum (FWHM) in the centre of the field of view (FOV) and 2.4 mm at 10-cm off-centre directions.[14]

A transmission scan of 6 min using a single ^{137}Cs source was performed immediately before radioligand injection. List mode data were acquired continuously for 123 min immediately after i.v. injection of the radioligand. Images were reconstructed with a series of frames (10 sec × 9, 15 sec × 2, 20 sec × 3, 30 sec × 4, 1 min × 4, 3 min × 4 and 6 min × 17).

In vitro autoradiography

The human brain used in this study was obtained from the National Institute of Forensic Medicine, Karolinska Institutet (Stockholm, Sweden). The brain had been removed at clinical autopsy and was handled in a manner similar to that previously described.[15] Horizontal sections including the striatum were selected for the binding experiments.

The sections were incubated for 20 min at RT with 0.2 MBq/mL [^{11}C]**1** in a TRIS buffer (50 mM; pH 7.4) containing sodium chloride (100 mM), and ascorbic acid (0.1% (w/v). The sections were then washed (same buffer) twice for 5 min each and briefly dipped in cold distilled water before being exposed to phosphor imaging plates (Fujifilm Plate BAS SR2025, Fujifilm, Tokyo, Japan). The phosphor imaging plates were scanned and the resulting images processed in a Fujifilm BAS-5000 phosphor imager (Fujifilm, Tokyo, Japan). Non-specific binding was estimated by simultaneous incubation with scopolamine (10 μM). Competition studies were also performed with two further reference ligands: (i) pirenzepine, 100 nM, a selective M1 receptor antagonist[16] (ii) donepezil, 100 nM, a selective Ach esterase inhibitor.[17]

Radiochemistry

[^{11}C]Methane was produced in a PETtrace medical cyclotron (General Electric Medical Systems, Uppsala, Sweden) using 16 MeV protons in the ^{14}N(p,α)^{11}C reaction on a mixture of nitrogen and hydrogen gas (10% hydrogen). [^{11}C]Methane was isolated from the target gas on a cryogenic trap and subsequently converted into [^{11}C]methyl iodide by radical iodination of [^{11}C]methane in a recirculation system as previously described elsewhere.[18] [^{11}C]Methyl triflate was in turn obtained by passing [^{11}C]methyl iodide through a heated column containing silver triflate impregnated on graphpac. Radiomethylation, purification and

formulation was performed using a computer controlled automated system (Scansys, Denmark). Semi-preparative HPLC was performed using a reversed-phase ACE 5 C-18L column (250 × 10 mm, 5 μm, Advanced Chromatography Technologies). The column outlet was connected with an UV absorbance detector (λ = 254 nm) in series with a detector for radioactivity. The radiochemical purity and identity of the formulated radiolabeled products were determined by analytic reverse phase HPLC using a ZORBAX Eclipse XDB-C18 column (150 × 3 mm, 5 μm; Agilent) and an UV absorbance detector (λ = 254 nm) in series with a β-flow detector for radioactivity (Beckman).

[^{11}C]**1**

[^{11}C]methyl triflate was transferred to a solution of **12** (0.5 mg, 1 μmol) and sodium hydroxide (6 μL, 0.5 M) in acetone (400 μL). After completed transfer, the crude reaction mixture was diluted with mobile phase acetonitrile:ammonium formate (0.1 M) 45:55 (600 μL) and purified by semi-preparative HPLC. The collected fraction containing the title compound (retention time = 8 minutes) was evaporated to dryness and re-dissolved in a solution of ethanol (5% v/v) in physiologically buffered saline (PBS, pH 7.4, 6.5 mL). The formulated product was sterilized by membrane filtration (0.22 μm, Millipore) to yield the final product in a solution ready for injection. [^{11}C]**1** co-eluted with an unlabeled reference standard of **1** on analytical HPLC. Its identity was further confirmed by tandem mass spectrometry (MS/MS) analysis of the carrier associated with **1** and comparison with an authentic reference standard. The radiochemical purity was >98% by HPLC and the determined average specific activity at injection was 147 GBq/μmol.

Associated content

Experimental details for the chemical synthesis.

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Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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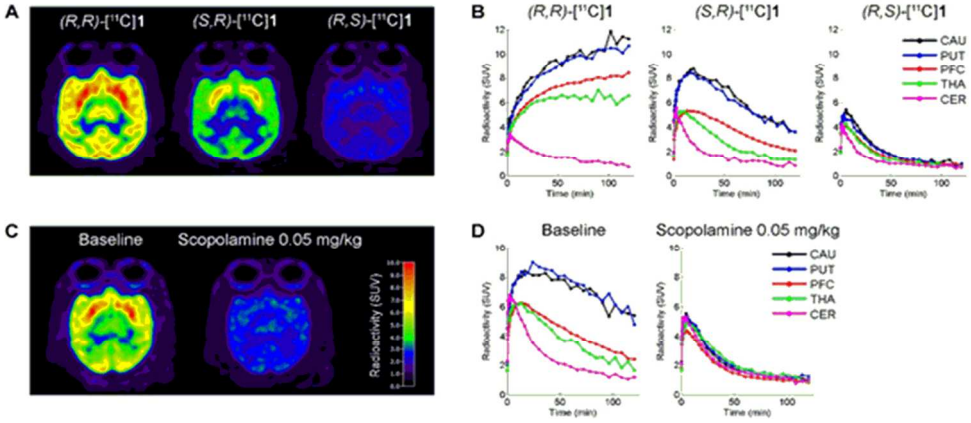

Graphical TOC

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