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Synthesis and in vivo evaluation of [^{11}C]SN003 as a PET ligand for CRF_1 receptors

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Abstract—Synthesis and evaluation of [*O*-methyl-^{11}C](4-methoxy-2-methylphenyl)[1-(1-methoxymethylpropyl)-6-methyl-1*H*-[1,2,3]triazolo[4,5-*c*]pyridin-4-yl]amine or [^{11}C]SN003 ([^{11}C]**6**), as a PET imaging agent for CRF_1 receptors, in baboons is described. 4-[1-(1-Methoxymethylpropyl)-6-methyl-1*H*-[1,2,3]triazolo[4,5-*c*]pyridin-4-ylamino]-3-methylphenol (**5**), the precursor molecule for the radiolabeling, was synthesized from 2,4-dichloro-6-methyl-3-nitropyridine in seven steps with 20% overall yield. The total time required for the synthesis of [^{11}C]SN003 is 30 min from EOB using [^{11}C]methyl triflate in the presence of NaOH in acetone. The yield of the synthesis is 22% (EOS) with >99% chemical and radiochemical purities and a specific activity of >2000 Ci/mmol. PET studies in baboon show that [^{11}C]**6** penetrates the BBB and accumulates in brain. No detectable specific binding was observed, likely due to the rapid metabolism or low density of CRF_1 receptors in primate brain.

1. Introduction

Corticotropin, releasing factor (CRF or CRH) is a naturally occurring 41-amino acid residue neuropeptide that regulates the hypothalamic–pituitary–adrenal (HPA) axis and is a neurotransmitter.[1] CRF modulates the endocrine, autonomic, behavioral, and immune responses to stress.[1] CRF acts on two subtypes (CRF_1 and CRF_2) and a CRF binding protein (CRF-BP).[2] CRF receptors are G-protein-coupled receptors (GPCR) and both the subtypes are found in the central nervous system (CNS), CRF_1 is found in cerebral cortex, cerebellum, olfactory bulb, medial septum, hippocampus, amygdala, and pituitary.[3] CRF_2 is predominantly limited to lateral septum, choroid plexus, and hypothalamus, but is widely expressed in peripheral tissues, including the heart, gastrointestinal tract, lung, skeletal muscle, and vasculature.[4] CRF-BP is widely distributed in CNS including hippocampus and amygdala.[4] Elevated expression of CRF_1 is implicated in the pathogenesis of neuropsychiatric disorders such as anxiety, mood disorders, and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, but direct evidence from in vivo studies is lacking.[5–11]

Development of a high-specific activity radiolabeled, pharmacologically selective CRF_1 receptor antagonist for positron emission tomography (PET) would make it possible to quantify binding to CRF_1 receptors in vivo, and permit us to study the pathophysiology of depression, anxiety, and neurodegenerative diseases. Currently there is no imaging agent available for the in vivo measurement of CRF_1 receptor in humans.[12–17] A number of non-peptide CRF_1 receptor antagonists that can specifically and selectively block the CRF_1 receptor subtype have been recently identified. To be effective in vivo, these molecules must have receptor subtype specificity (a high CRF_1/CRF_2 affinity ratio), aqueous solubility, good oral bioavailability, and rapid permeability across the blood–brain barrier (BBB).[18] We have selected SN003 as a potential PET ligand based on its high binding affinity (K_i = 4 nmol/L) and in vivo effect on CRF_1 receptors.[19] [^{3}H]SN003 binding to rat cortex and human cell membranes demonstrates K_D values of 4.8 and 4.6 nM, respectively, and B_{max} values of 0.142 and 7.42 pmol/mg protein.[19] Moreover, the distribution of [^{3}H]SN003 binding sites is consistent with the expression

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pattern of CRF_1 receptors in rat brain regions.[19] This attractive in vitro profile coupled with a favorable calculated lipophilicity of c log P = 3.3 (calculated with ACD/log P DB program) to penetrate BBB, and a potential site for [^{11}C]-labeling prompted us to develop [^{11}C]SN003 as a candidate ligand for the in vivo quantification of CRF_1 receptors.

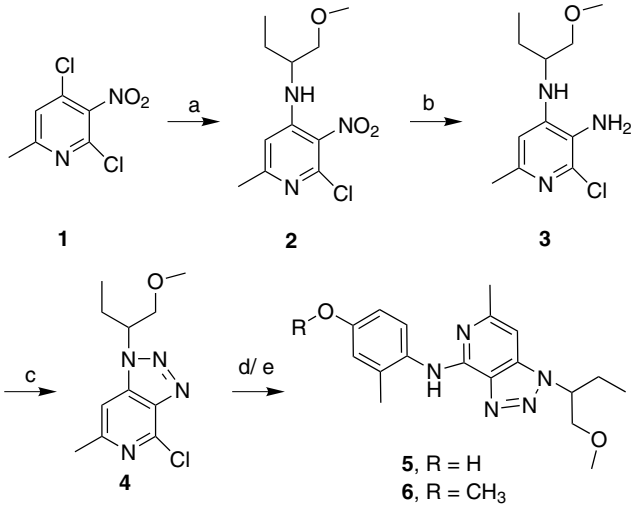
2. Results and discussion

2.1. Chemistry

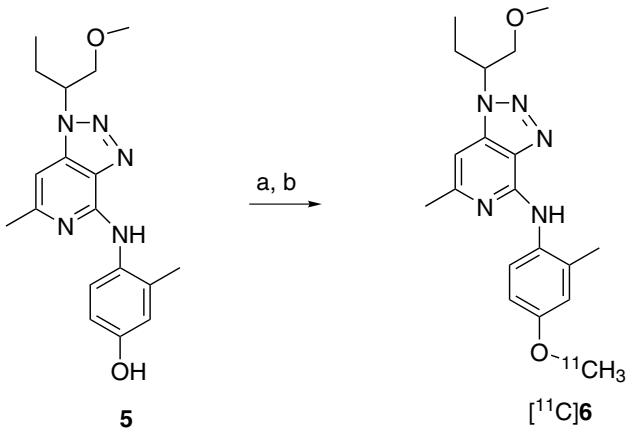
The synthesis of 4-[1-(1-methoxymethylpropyl)-6-methyl-1*H*-[1,2,3]triazolo[4,5-c]pyridin-4-ylamino]-3-methylphenol (**5**), the precursor molecule for the radiolabeling, and SN003 standard (**6**) was achieved from 2,4-dichloro-6-methyl-3-nitropyridine (**1**) in four steps with an overall yield of 20% (Scheme 1). Condensation of pyridine **1** with 1-(methoxymethyl)propylamine in the presence of DIPEA provided (2-chloro-6-methyl-3-nitropyridin-4-yl)(1-methoxymethylpropyl)amine (**2**) in 48% yield. Stannous chloride mediated reduction of nitro compound **2** provided 2-chloro-*N*4-(1-methoxymethylpropyl)-6-methylpyridine-3,4-diamine (**3**) in 96% yield. Diazotization of diamine **3**, followed by acid catalyzed ring closure, yielded 4-chloro-1-(1-methoxymethylpropyl)-6-methyl-1*H*-[1,2,3]triazolo-[4,5-*c*]pyridine (**4**) in 98% yield.[20] The radiolabeling precursor was synthesized by reacting compound **4** with 4-amino-3-methylphenol in the presence of NaHMDS as base in 41% yield. Unlabeled SN003 was synthesized from **4** by coupling with 4-methoxy-2-methylphenylamine in 51% yield.

2.2. Radiochemistry

The labeling reaction was initially modeled with non-radioactive methyl triflate and the reaction condition was optimized using NaOH as the base in THF. Under these conditions unlabeled SN003 (**6**) was obtained in 90% yield. Labeling experiments with precursor **5** using [^{11}C]MeOTf in acetone using NaOH provided [^{11}C]**6**. The radiolabeled product was separated from the reaction mixture by reverse-phase high performance liquid chromatography (RP-HPLC) with an average yield of 22 ± 4% (EOS, Scheme 2, n = 6) (Scheme 2). The chemical identity of [^{11}C]**6** was confirmed by co-injection with an authentic sample of standard **6** on analytical RP-HPLC. The specific activity was calculated based on a standard mass curve using HPLC technique. The chemical and radiochemical purities of [^{11}C]**6** was found to be >99% with a specific activity 2000 ± 400 Ci/mmol (EOB, n = 6). The average time required for the [^{11}C]-labeling was 30 min (EOB). The partition coefficient (log $P_{o/w}$) of [^{11}C]**6** was determined by standard shake flask method[21] and found to be 3.1.



Scheme 1. Synthesis of SN003 and radiolabeling precursor. Reagents and conditions: (a) 1-(methoxymethyl)propylamine, DIPEA, CH_3CN, rt, reflux, 48%; (b) $SnCl_2{\cdot}2H_2O$, EtOH, 70 °C, 1h, 96%; (c) $NaNO_2$, aq CH_3COOH, CH_2Cl_2, 0 °C, 98%; (d) R = H; NaHMDS, THF, 4-amino-3-methylphenol, −78 °C to rt, 41%; (e) R = CH_3; NaHMDS, THF, 4-methoxy-2-methylphenylamine, −78 °C to rt, 51%.



Scheme 2. Radiosynthesis of [^{11}C]SN003. Reagents and conditions: (a) 5 M NaOH, acetone, [^{11}C]CH_3OTf, rt to 60 °C, 4 min; (b) semipreparative HPLC purification, 22% (EOS).

3. In vivo imaging in baboon

As is evident from Figure 1A, the PET studies in baboon show that the tracer penetrates BBB and accumulates in brain. The time–activity curves of several brain regions were examined and no regional specific binding was detected (Figs. 1B and 2). The regional distribution is found to be homogeneous across all brain regions after 10 min and a fast washout of radioactivity was observed (Fig. 1B). The radioactive metabolites were determined by HPLC technique. Only polar metabolites were found

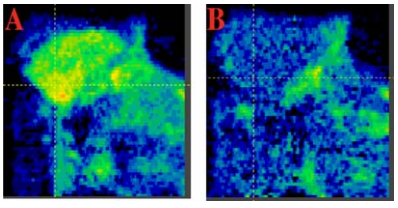


Figure 1. PET images of [^{11}C]SN003 in baboon. (A) Sagittal view of early frames (sum of first 20 min); (B) later frames (sum of last 40 min).

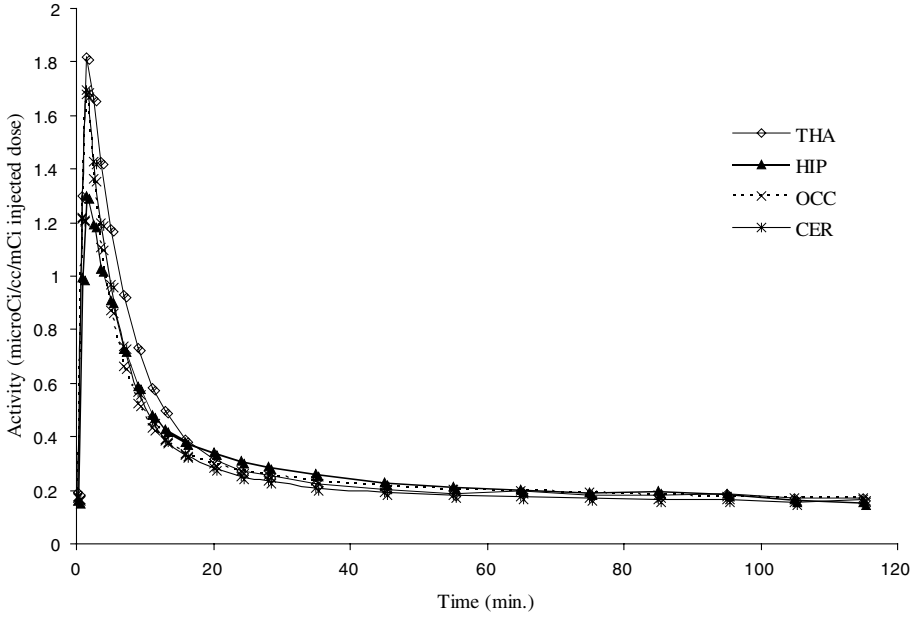


Figure 2. Time–activity curves of the radioactivity in baboon after the injection of [^{11}C]SN003. CER, cerebellum; OCC, occipital cortex; THA, thalamus; HIP, hippocampus.

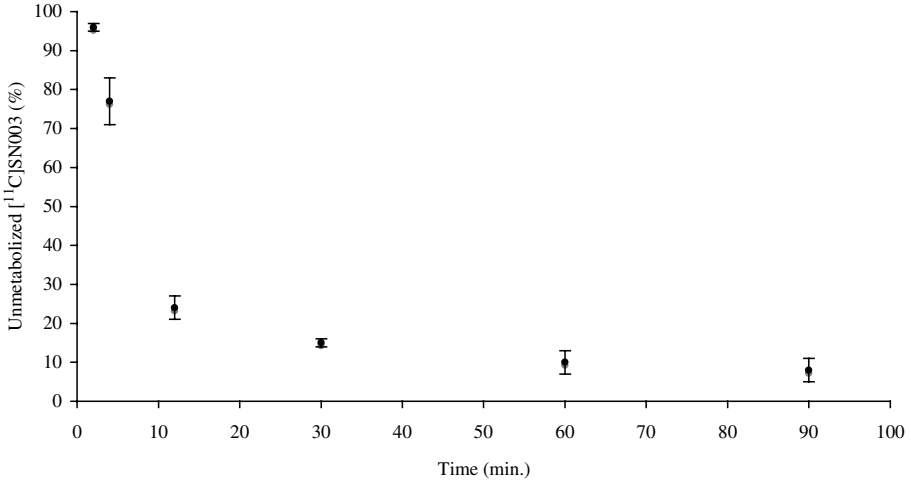


Figure 3. Unmetabolized parent fraction of [^{11}C]SN003 in baboon plasma. Filled circles represent the mean fraction in six determinations. Error bars are standard deviations.

in baboon plasma and the % of unmetabolized fractions were 96%, 77%, 24%, 15%, 10%, and 8% at 2, 4, 12, 30, 60, and 90 min, respectively (Fig. 3). The metabolite data indicate that [^{11}C]**6** undergoes rapid metabolism in baboon that would contribute to its poor specific binding.

4. Conclusion

The radiosynthesis of [^{11}C]SN003, a CRF_1 antagonist, has been achieved. Total time required for the synthesis of [^{11}C]SN003 is 30 min from EOB using [^{11}C]methyl triflate in acetone, with a 22% yield at EOS based on [^{11}C]MeOTf. The chemical and radiochemical purities are >99% and the specific activity is >2000 Ci/mmol. In vivo PET imaging studies in baboon revealed that [^{11}C]SN003 penetrated the BBB, but no regional variation in total binding was observed possibly due to rapid metabolism or the small number of CRF_1 receptors in baboons.

5. Materials and methods

5.1. General

The commercial chemicals used in the synthesis were purchased from Sigma–Aldrich Chemical Co., Fisher Scientific Inc. or Lancaster and were used without further purification. Melting points were determined on a Fisher Scientific Melting point apparatus. ^{1}H NMR

spectra were recorded on a Bruker PPX 300 and 400 MHz spectrometer. Spectra were recorded in $CDCl_3$ or CD_3OD and chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS). The mass spectra were recorded on JKS-HX 11UHF/HX110 HF Tandem Mass Spectrometer in the fast atom bombardment (FAB+) mode. Semipreparative HPLC analyses were performed using a Waters 1525 HPLC system, Phenomenex, Prodigy ODS(3) 10×250 mm, 10 mm, 10 μm column using 40:60 (acetonitrile/0.1 M ammonium formate solution), 10 ml/min flow rates and for analytical studies Phenomenex, Prodigy ODS(3) 4.6×250 mm, 5 μm column using 40:60 (acetonitrile/0.1 M ammonium formate solution), 2 mL/min as flow rate were used. Flash chromatography was performed on silica gel (Fisher 200–400 mesh) using the solvent system indicated in the experimental procedure for each compound. [^{11}C]Methyl triflate was synthesized in the Radioligand Laboratory of Columbia University by transferring [^{11}C]methyl iodide through a glass column containing silver triflate (AgOTf) at 200 °C. The radiochemical and chemical purities were analyzed by RP-HPLC with PDA and NaI detectors. Partition coefficient determination was performed with a Packard Instruments Gamma Counter (Model E5005). Metabolite analyses were performed using Phenomenex (C18, 10×250 mm, 5 μm) column using a mobile phase 40:60 (acetonitrile/0.1 M ammonium formate solution). The free fractions and metabolites were measured using Packard Instruments Gamma Counter (Model E5005).

5.2. Chemistry

5.2.1. 2-Chloro-6-methyl-3-nitropyridin-4-yl-(1-methoxymethylpropyl)amine (2). A solution of the aryl chloride **1** (1.68 g, 8.14 mmol) and 1-(methoxymethyl)propylamine (840 mg, 8.14 mmol) in CH_3CN (15 mL) was treated with DIPEA (1.7 mL, 9.77 mmol, dropwise addition) at room temperature (rt). The reaction mixture was stirred at rt for 12 h and then heated to reflux for 3 h. Excess CH_3CN was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (30 mL), washed with water (20 mL). The aqueous layer was extracted with dichloromethane (2× 20 mL) and the combined organic layer was dried over $MgSO_4$, concentrated under high vacuum, and column chromatographed (75:25 hexane/EtOAc) to yield the product **2** as a pale yellow solid (1.07 g, 48%). Mp 63 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.75 (d, J = 7.5 Hz, 1H), 6.49 (s, 1H), 3.62–3.52 (m, 1H), 3.44 (d, J = 4.8 Hz, 2H), 3.36 (s, 3H), 2.42 (s, 3H), 1.83–1.68 (m, 1H), 1.66–1.52 (m, 1H), 0.98 (t, J = 7.4 Hz, 3H); HRMS calcd for $C_{11}H_{17}ClN_3O_3$ (MH^+): 274.0958; found: 274.0961.

5.2.2. 2-Chloro-*N*4-(1-methoxymethylpropyl)-6-methylpyridine-3,4-diamine (3). A solution of the nitro compound **2** (608 mg, 2.22 mmol) was dissolved in dry ethanol (5 mL) and treated portionwise with $SnCl_2 \cdot 2H_2O$ (2.50 g, 11.10 mmol) at rt. The reaction mixture was heated at 70 °C for 1 h, concentrated under reduced pressure, and neutralized with saturated $NaHCO_3$ solution, and ethyl acetate was added. The resultant suspension was filtered to remove the solids, the residue was washed with EtOAc, and the aqueous layer was extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated. Recrystallization of the crude residue from hexane: Et_2O gave the desired amine **3** as a colorless solid (520 mg, 96% yield). Mp: 127 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.30 (s, 1H), 4.41 (br s, 1H), 3.52–3.42 (m, 3H), 3.38 (s, 3H), 2.38 (s, 3H), 1.80–1.56 (m, 2H), 0.99 (t, J = 7.4 Hz, 3H). HRMS calcd for $C_{11}H_{19}ClN_3O$ (MH^+): 244.1217; found: 244.1203.

5.2.3. 4-Chloro-1-(1-methoxymethylpropyl)-6-methyl-1*H*-[1,2,3]triazolo[4,5-*c*]pyridine (4). $NaNO_2$ (92 mg, 1.34 mmol) was added portionwise to a solution of the amine **3** (182 mg, 0.75 mmol) in aqueous acetic acid (2 mL, 50%) and dichloromethane (2 mL) at 0 °C. After stirring for 30 min at 0 °C, the reaction mixture was allowed to warm to rt and was stirred for further 1 h. The solution was then cooled and neutralized with solid $NaHCO_3$. Water (4 mL) was then added and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated to give the product **4** as a viscous liquid (187 mg, 98%), which was used for the next step without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.25 (s, 1H), 4.81–4.69 (m, 1H), 3.92 (dd, J = 9.9, 8.0 Hz, 1H), 3.83 (dd, J = 4.2, 9.9 Hz, 1H), 3.26 (s, 3H), 2.68 (s, 3H), 2.30–2.22 (m, 1H), 2.19–2.06 (m, 1H), 0.86 (t, J = 7.4 Hz, 3H); HRMS calcd for $C_{11}H_{15}ClN_4O$ (MH^+): 255.1013; found: 255.1028.

5.2.4. 4-[1-(1-Methoxymethylpropyl)-6-methyl-1*H*-[1,2,3]-triazolo[4,5-*c*]pyridin-4-ylamino]-3-methylphenol (5). A solution of the aryl chloride **4** (73 mg, 0.28 mmol) was dissolved in THF (2 mL) and treated with NaHMDS (650 μL, 0.65 mmol, 1.0 M in THF, dropwise addition) at −78 °C. A solution of 4-amino-3-methylphenol (60 mg, 0.49 mmol) in THF (1 mL) was then introduced dropwise and the reaction mixture was allowed to warm to rt and was stirred for further 2 h. Water was added and the mixture was extracted with EtOAc (3× 5 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, concentrated under reduced pressure without heating, and column chromatographed (30% EtOAc in hexanes) to yield the product **5a** as a colorless solid (40 mg, 41%); Mp 135–137 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.43 (d, J = 9.3 Hz, 1H), 6.52–6.43 (m, 4H), 4.65–4.57 (m, 1H), 3.87 (dd, J = 9.9, 7.9 Hz, 1H), 3.75 (dd, J = 4.7, 9.9 Hz, 1H), 3.22 (s, 3H), 2.44 (s, 3H), 2.19 (s, 3H), 2.12–1.97 (m, 2H), 0.79 (t, J = 7.4 Hz, 3H). HRMS calcd for $C_{18}H_{24}N_5O2$ (MH^+): 342.1930; found: 342.1933.

5.2.5. (4-Methoxy-2-methylphenyl)-[1-(1-methoxymethylpropyl)-6-methyl-1*H*-[1,2,3]triazolo[4,5-*c*]pyridin-4-yl]amine (6). A solution of the aryl chloride **4** (33 mg, 0.13 mmol) was dissolved in THF (2 mL) and treated with NaHMDS (280 μL, 0.28 mmol, 1.0 M in THF) at −78 °C. A solution of 4-methoxy-2-methylphenylamine (35 mg, 0.26 mmol) in THF (1 mL) was then introduced dropwise and the reaction mixture was allowed to warm to rt and was stirred for further 2 h. Water was added

and the mixture was extracted with EtOAc (3×5 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, concentrated, and column chromatographed (25% EtOAc in hexanes) to yield the product **6** as a viscous liquid (23 mg, 51%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.06 (d, J = 9.6 Hz, 1H), 7.33 (br s, 1H), 6.83–6.80 (m, 2H), 6.61 (s, 1H), 4.73–4.66 (m, 1H), 3.94 (dd, J = 9.9, 7.9 Hz, 1H), 3.84 (d, J = 4.8 Hz, 1H), 3.82 (s, 3H), 3.29 (s, 3H), 2.49 (s, 3H), 2.38 (s, 3H), 2.24–2.12 (m, 1H), 2.11–2.00 (m, 1H), 0.84 (t, J = 7.4 Hz, 3H). HRMS calcd for $C_{19}H_{26}N_5O_2$ (MH^+): 356.2087; found: 356.2097.

5.3. Radiochemistry

Radiosynthesis of [^{11}C]SN003: [^{11}C]MeOTf[22] was trapped into an acetone (400 μL) solution containing 0.5 mg of **5** and 10 μL of 5 N NaOH at rt for 5 min. At the end of the trapping, the reaction mixture was heated on a water bath at 60 °C for 4 min and then directly injected into a semipreparative RP-HPLC and eluted with acetonitrile/0.1 M ammonium formate solution (50:50) at a flow rate of 10 mL/min. The product fraction with a retention time of 10–11 min based on γ-detector was collected, diluted with 100 mL of deionized water, and passed through a classic C-18 Sep-Pak cartridge. Reconstitution of the product in 1 mL of absolute ethanol afforded [^{11}C]SN003 (67% yield based on [^{11}C]CH_3OTf at EOB and 22% based on EOS). A portion of the ethanol solution was analyzed by analytical HPLC (Mobile phase: acetonitrile/0.1 M ammonium formate solution 60:40, flow rate: 2 mL/min, retention time: 6.4 min) to determine the specific activity and radiochemical purity.

5.4. Partition coefficient measurement

Partition coefficient (log P) of the [^{11}C]**6** was measured by mixing 0.1 mL of the radioligand formulation with 5 g each of 1-octanol and freshly prepared PBS buffer (pH 7.4) in a culture tube.[21] The culture tube was shaken mechanically for 5 min followed by centrifugation for 5 min. Radioactivity per 0.5 g each of 1-octanol and aqueous layer was measured using a well counter. The partition coefficient was determined by calculating the ratio of counts/g of 1-octanol to that of buffer. 1-Octanol fractions were repeatedly portioned with fresh buffer to get consistent values for partition coefficient. All the experimental measurements were performed in triplicate.

5.5. PET studies in baboons

A series of PET studies were done on two baboons (*Papio anubis*) with an ECAT EXACT HR+ scanner (Siemens, Knoxville, TN). Each animal was scanned on six separate occasions, with two tracer injections on each occasion. For each scanning session, the fasted animal was immobilized with ketamine (10 mg/kg, im) and anesthetized with 1.5–2.0% isoflurane via an endotracheal tube. Core temperature was kept constant at 37 °C with a heated water blanket. An iv infusion line with 0.9% NaCl was maintained during the experiment and used for hydration and radiotracer injection. An arterial line was placed for obtaining arterial samples for determination of the input function for quantification purposes. In each scanning session, the head was positioned so that the brain was in the center of the field of view, and a 10 min transmission scan was performed before the first tracer injection. For each scan 5.0 ± 0.5 mCi of [^{11}C]**6** was injected as an iv bolus and emission data were collected for 120 min in 3D mode with the following time frames: 2 × 0.5, 3 × 1, 5 × 2, 4 × 4, and 9 × 10 min. Plasma samples were taken every 10 s for the first 2 min, using an automatic system, and thereafter manually for a total of 34 samples over 2 h.

5.6. Determination of unchanged radioligand in plasma

The percentage of radioactivity in plasma as unchanged [^{11}C]**6** was determined by HPLC method. Blood samples were taken at 2, 6, 12, 30, 60, and 90 min after radioactivity injection for metabolite analyses. The supernatant liquid (0.5 mL) obtained after centrifugation of the blood sample at 2000 rpm for 1 min was transferred (0.5 mL) into a tube and mixed with acetonitrile (0.7 mL). The resulting mixture was vortexed for 10 s and centrifuged at 14,000 rpm for 4 min. The supernatant liquid (~1 mL) was removed, the radioactivity measured in a well-counter, and the majority (0.8 mL) was subsequently injected onto the HPLC column and eluted with a mobile phase acetonitrile/0.1 M ammonium formate (60:40) solution at 2 mL/min (Phenomenex C18, 10 × 250 mm, 5 μm). The metabolite and free fractions were collected using a Bioscan gamma detector and all the acquired data then subjected to correction for background radioactivity and physical decay to calculate the percentage of [^{11}C]SN003 in the plasma at different time points.

5.7. Image processing and analysis

The PET data were reconstructed with attenuation correction using the transmission data, and scatter correction was done using model-based scatter correction.[23] The reconstruction filter and estimated image filter were Shepp 0.5, the axial (Z) filter was all pass 0.4, and the zoom factor was 4.0. The final image resolution at the center of the field of view was 5.1 mm FWHM.[24] A T1-weighted magnetic resonance image (MRI) of the animal's head was acquired on a GE 1.5-T Signa Advantage system. Regions of interests were drawn on the MRI using the MEDX software (Sensor Systems, Inc., Sterling, VA). ROIs included cerebellum, hippocampus, occipital cortex, and thalamus. The PET data were co-registered to the MRI using the software AIR,[25] and time–activity curves (TACs) were generated for each ROI.

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