Published in final edited form as:

Org Biomol Chem. 2019 June 05; 17(22): 5586–5594. doi:10.1039/c9ob00893d.

Development of a Carbon-11 PET Radiotracer for Imaging TRPC5 in the Brain†

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Abstract

The transient receptor potential channels subfamily member 5 (TRPC5) is a calcium permeable cation channel widely expressed in the brain. Accumulating evidence indicates that it plays a crucial role in psychiatric disorders including depression and anxiety. Positron emission tomography (PET) combining with a TRPC5 specific radioligand may provide a unique tool to investigate the functions of TRPC5 in animal disease models to guide drug development targeting TRPC5. To develop a TRPC5 PET radiotracer, the potent TRPC5 inhibitor **HC608** was chosen for C-11 radiosynthesis through the *N*-demethyl amide precursor **7** reacting with [¹¹C]methyl iodide. Under optimized conditions, [¹¹C]**HC608** was achieved with good radiochemical yield (25 ± 5%), high chemical and radiochemical purity (>99%), and high specific activity (204–377 GBq μmol⁻¹, decay corrected to the end of bombardment, EOB). *In vitro* autoradiography study reveals that [¹¹C]**HC608** specifically binds to TRPC5. Moreover, initial *in vivo* evaluation of [¹¹C]**HC608** performed in rodents and microPET study in the brain of non-human primates further demonstrate that [¹¹C]**HC608** was able to penetrate the blood brain barrier and sufficiently accumulate in the brain. These results suggest that [¹¹C]**HC608** has potential to be a PET tracer for imaging TRPC5 *in vivo*.

Graphical Abstract

A potent carbon-11 PET tracer targeting TRPC5 was radiosynthesized successfully and the preliminary evaluation in rodents and nonhuman primate were performed.

†Electronic Supplementary Information (ESI) available. ¹H NMR and ¹³C NMR spectra. See DOI: 10.1039/x0xx00000xtuz@mir.wustl.edu.

Conflicts of interest

There are no conflicts to declare.

Introduction

Depression is a common cataclysmic psychiatric disorder widespread among world populations and it is one of the leading causes of global medical disability and suicide^{1,2}. The management of depression remains a challenge, due to lack of effective treatments. Usually, 2–4 weeks are required before antidepressant medications display efficacy, with approximately 30% of patients failing treatment with several different medications³. Therefore, new pharmacological targets with faster therapeutic response are urgently needed. Transient receptor potential channels subfamily member 5 (TRPC5) is a Ca^{2+} permeable cation channel that belongs to the transient receptor potential (TRP) superfamily⁴ and it is widely expressed in the brain⁵. In response to numerous physiological or pathological stimuli, TRPC5 channels function as either homomeric or heteromeric channel complexes together with TRPC1 or TRPC4 regulating intracellular free Ca^{2+} concentration, which subsequently promotes signal transduction, gene expression and cellular phenotype^{6–8}. Compelling evidence indicates that TRPC5 plays crucial roles in depression and other mental disorders^{9–11}. TRPC5 knock-out mice exhibit increased exploratory behaviours in both open field test and elevated plus maze (EPM), and have markedly reduced symptoms of depression and anxiety⁹. Nevertheless, the precise functions and mechanisms of the TRPC5 channel in relevant neurological diseases and psychiatric disorders remain inadequately understood. One of the key challenges is the lack of highly potent and specific pharmacological probes to modulate the channel action *in vivo* in living animals.

In recent years, several TRPC5 modulators, including activators such as (–)-Englerin A¹², BTD¹³, Riluzole¹⁴ and inhibitors such as ML204¹⁵, clemizole¹⁶, AC 1903¹⁷ have been reported (Fig.1). However, these compounds have limitations such as their low potency with IC_{50} value in the micromolar range or have poor selectivity for TRPC5 compared to other TRP channels. More recently, the discovery of 7-(4-chlorobenzyl)-1-(3-hydroxypropyl)-3-methyl-8-(3-trifluoro-methoxy)-phenoxy)-3,7-dihydro-1H-purine-2,6-dione, **HC608**¹⁸, was a remarkable accomplishment (Fig.1). Compound **HC608**, also known as Pico145 or C31¹⁹, has an IC_{50} value of 6.2 nM towards TRPC5, and weaker binding to TRPC4 with an IC_{50} value of 32.5 nM²⁰. Importantly, **HC608** can distinguish between closely related channels and has no binding activities toward other TRP channels including TRPC3 and 6, TRPV (vanilloid) 1 and 4, TRPA (ankyrin) 1, and TRPM (melastatin) 2 and 8¹⁹. In addition, 7-(4-chlorobenzyl)-8-(3-chlorophenoxy)-1-(3-hydroxypropyl)-3-methyl-3,7-dihydro-1H-purine-2,6-dione, **HC070** (Fig.1), a structurally close molecule of **HC608** produces anxiolytic and antidepressant effects in mice²⁰. These valuable findings suggest that either

[¹¹C]HC608 or [¹¹C]HC070 could be a promising PET radiotracer targeting TRPC5. Here we reported our efforts on synthesis/radiosynthesis of [¹¹C]HC608 and initial evaluation of [¹¹C]HC608 in rodents and microPET imaging of [¹¹C]HC608 in the brain of nonhuman primate to explore the feasibility of [¹¹C]HC608 to be a PET radiotracer for imaging TRPC5 *in vivo*.

Results and discussion

Chemistry and radiochemistry

The standard reference compound HC608 and its analogue HC070 were prepared according to the reported procedure with necessary modification¹⁹.

HC608 possesses a *N*-methyl group on Xanthine skeleton which facilitates utilizing a conventional method to introduce carbon-11 via *N*-alkylation of the desmethyl precursor, 7-(4-chlorobenzyl)-1-(3-hydroxypropyl)-8-(3-(trifluoromethoxy) phenoxy)-3,7-dihydro-1H-purine-2,6-dione (**7**), reacting with [¹¹C]triflate or [¹¹C]methyl iodide. As illustrated in Scheme 1, the synthesis of precursor **7** was achieved by starting with the commercially procured Xanthine **1**. Using a solution of bromine to convert compound **1** to 8-bromoxanthine **2**, followed by *N*-alkylation on imidazole ring of purine scaffold, intermediate **3** was recovered with high yield. Although two positions in the structure of **3** could be *N*-alkylated, it was found when (2-(chloromethoxy)ethyl)trimethylsilane was added into the reaction vial at lower temperature, intermediate **4** was generated as the major product. With the 2-(trimethylsilyl)-ethoxymethyl (SEM) group blocked, the tetrahydropyranyl (THP) ether could be easily added to the Xanthine ring to furnish **5** with a 72% yield. Intermediate **6** was made with a 75% yield via etherification of aryl bromide **5** with 3-(trifluoromethoxy) phenol in DMF in the presence of K₂CO₃ at 80 °C. Concentrated hydrochloride solution acted as a deprotection agent to remove SEM and THP groups simultaneously to finalize the synthesis of **7**.

To radiosynthesize [¹¹C]HC608 starting from precursor **7**, multiple experimental conditions were tested for generating the *N*-[¹¹C]methyl group. Radioactive [¹¹C]CH₃I or [¹¹C]methyl triflate ([¹¹C]CH₃OTf), solvent, amount of precursor, and different bases were determined and summarized in Table 1. The optimizing conditions include: 0.6–0.8 mg of precursor **7**, 300 μL of DMF, 2.0 mg of dry K₂CO₃, heating for 5 min at 90 °C (Table 1, entry 6). Under these conditions, it took 50–60 min from bombardment of [¹¹C]CO₂ to the formulation of the injection dose of [¹¹C]HC608 with good radiochemistry yield of 25 ± 5% (decayed to the end of bombardment (EOB), *n* > 10), high radiochemical purity (> 99%) and high specific activity (SA = 204–377 GBq μmol^{−1} (EOB)). In the process of making this radiotracer, it was observed that the injection dose of [¹¹C]HC608 was sticky and a significant amount of radioactivity might be retained within the syringe when transferred into other vial or injected into animals. To avoid excessive product retention in the syringe, 5% polyethylene glycol (15)-hydroxystearate (Kolliphor® HS15), a potent non-ionic solubilizer and emulsifying agent, was applied for formulating the injection dose²⁰.

Ex vivo Biodistribution study of [^{11}C]HC608 in rats

To investigate the kinetics and tissue distribution of [^{11}C]HC608 in rodents, we performed *ex vivo* biodistribution study using male adult Sprague Dawley (SD) rats. After injection of radiotracer, animals were euthanized at 5, 30 and 60 min ($n = 4$ rats/group). The tissue uptake of the radioactivity was presented as the percentage of injected dose per gram wet tissue (%ID/gram) in Table 2. Among the selected tissues, heart, lung, pancreas, kidney and liver have relative high uptake (>1.5 %ID/gram) at 5 min post injection. The radioactivity washed out quickly from all tissues except liver; at 60 min, liver retained 2.22 %ID/gram radioactivity, while all other tissue tracer uptake was <1.0 % ID/gram. The initial brain uptake (%ID/gram) was moderate with 0.51 at 5 min, 0.37 at 30 min, and 0.25 at 60 min. The brain uptake ratio at 5 min versus 60 min was 2.04, suggesting that [^{11}C]HC608 penetrates the blood brain barrier and has sufficient accumulation in the rat brain.

In vitro autoradiography (ARG) and H&E staining study

To check the distribution of [^{11}C]HC608 in the TRPC5-enriched brain regions of interests, *in vitro* autoradiography study was performed using 20 μm SD rat brain frozen sections. Brain sections were incubated with [^{11}C]HC608 at different dosages for 30 min at room temperature. For a blocking study, HC070, a compound structurally similar to [^{11}C]HC608, with IC_{50} values of 9.3 and 46.0 nM for TRPC5 and 4, respectively, was utilized at 1.0 μM of concentration. After 30 min incubation, sections were washed under low and high stringent washing conditions.

ARG signals on the rat brain sections with [^{11}C]HC608 at 2.22 MBq/slide or higher were at saturation levels whether low or high stringent washing conditions were employed (data not shown). Tracer dosages at 0.74, 1.11, and 1.48 MBq/slide gave good ARG signals on rat brain sections as shown in Fig.2. Positive ARG signal lights up essentially all major regions with strong ARG signals on cortex, hippocampus, midbrain, and brain stem regions. Tracer dose at 1.11 MBq/slide provided the best results, while dose at 1.48 MBq/slide was too high, doses at 0.74 MBq/slide yielded poor ratios between ARG signals with and without the blocker regardless of washing conditions. In addition, high stringent washing condition has less tissue off and lower background ARG signal. Consequently, we set 1.11 MBq/slide with high stringent washing condition as optimized parameters for ARG study. Remarkably, under this condition, the presence of the blocking agent HC070 (1 μM) significantly reduced the *in vitro* ARG signal, and the ratio of the average signal intensity for baseline over blocking slide was 2.44:1 (~60% reduce). Over all, our ARG studies suggested: a) TRPC4/5 channels on the frozen rat brain tissue remain biologically active, b) [^{11}C]HC608 retains strong binding activities to TRPC5 channels on rat brain sections, c) HC070 significantly reduces [^{11}C]HC608 uptake, demonstrating specific binding of [^{11}C]HC608 to TRPC5 in the brain slides.

After the ARG experiment, rat brain sections were co-stained with Haematoxylin and Eosin (H&E) to show their anatomic structure. The matching alignments between ARG signal and anatomic structures on sagittal, horizontal, and coronal sections are illustrated in Fig.3. High ARG signals in cortex, hippocampus, thalamus, mid brain, striatum, and brain stem regions were observed. These findings recapitulate the findings in earlier reports^{21–23}.

The short half-life of Carbon-11 (20.3 min) limits the radioactivity on the slides during the incubation process for autoradiography. To make sure sufficient signal was generated when performing the *in vitro* autoradiography using [^{11}C]HC608 for brain slides, we started with a high tracer dose at 6.29 MBq/slide. Optimal outcomes was achieved with a radioactive dose of the ^{11}C -tracer at 1.11 MBq; no more than 1.48 MBq/slide are needed. The high lipophilicity of [^{11}C]HC608 is one of our concerns for autoradiography studies since brain tissue is rich in lipids, and lipophilic [^{11}C]HC608 may require stringent washing condition, such as washing buffers with a high concentration of alcohol to reduce nonspecific background of ARG signals. However, the stringent washing buffers may also disrupt the protein structure conformation and components of TRPC channels, and suppress the ARG signal of specific binding on rat brain sections. Our optimized *in vitro* ARG protocol indicates that washing buffer with alcohol up to 30% is compatible for detecting binding of [^{11}C]HC608 to TRPC5 channels on rat brain sections.

Non-human primate (NHP) microPET study

To further investigate the ability to cross blood brain barrier and the *in vivo* kinetic of [^{11}C]HC608 in the NHP brain, a microPET study was conducted on a male adult cynomolgus macaque. As displayed in Fig.4, the brain standardized uptake value (SUV) reached a maximum (~1.1) at 6–10 min post tracer injection, followed by a fast washout from the NHP brain. The uptake (SUV) ratio of [^{11}C]HC608 at 8 min versus 120 min reached 2.75-fold. These microPET data suggest that [^{11}C]HC608 crosses the blood brain barrier and possesses a fast washout kinetic in the NHP brain although the brain uptake is relatively low as a brain PET radiotracer.

Conclusions

In conclusion, we have successfully synthesized the first TRPC5 PET radioligand, [^{11}C]HC608, with good radiochemical yield, high chemical and radiochemical purity, and high specific activity. Our initial *ex vivo* biodistribution and *in vitro* autoradiography studies in rodents and microPET study in the NHP brain suggest that [^{11}C]HC608 specifically binds to TRPC5 in the rat brain sections and penetrates the blood brain barrier and accumulates in the brain of rats and NHP. Together, these findings indicate that [^{11}C]HC608 has a great potential to be developed into a PET radiotracer for *in vivo* quantification of TRPC5 expression in the brain. Further investigation of [^{11}C]HC608 in animal models of neurological diseases will advance our understanding the functions of TRPC5 in the brain.

Experimental

General

All the starting materials, and solvents were purchased commercially and used as received, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 F₂₅₄ (EMD Chemicals Inc, Billerica, MA) Flash column chromatography was conducted using 230–400 mesh silica gel (SiliCycle Inc, Quebec, Canada). Melting points were determined using MEL-TEMP 3.0 apparatus without correction. ^1H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Varian spectrometer with CDCl_3 as

solvent and tetramethylsilane (TMS) as the internal standard. All chemical shift values are reported in parts per million (ppm) and were calibrated using a residual undeuterated solvent as an internal reference (CDCl₃: δ 7.26 ppm). Coupling constants (J) are reported in units of Hertz (Hz). The following abbreviations are used to describe multiplicities – s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br. (broad). High resolution mass spectra (HRMS, m/z) was acquired by a Bruker MaXis 4G Q-TOF mass spectrometer with electrospray ionization source.

All animal experiments were conducted under Washington University's Institutional Animal Care and Use Committee (IACUC)-approved protocols in accordance with the US National Research Council's Guide for the Care and Use of Laboratory Animals.

Chemistry

The standard compound **HC608** and block agent **HC070** were synthesized via 4 steps according to a modified procedure reported¹⁹. Briefly, 1-(bromomethyl)-4-chlorobenzene (1.25 g, 6.1 mmol) was added to a suspension of 8-bromo-3-methyl-1H-purine-2,6-dione (1.5 g, 6.1 mmol) and K₂CO₃ (0.85 g, 6.1 mmol) in DMF (10 mL). The mixture was stirred at rt for 1.5 hours before another equivalent of K₂CO₃ (0.85 g, 6.1 mmol) and 2-(3-bromopropoxy)tetrahydro-2H-pyran (1.1 mL, 6.4 mmol) were added, and the mixture was stirred overnight at 60 °C. After cooling, the mixture was poured into water (50 mL) and extracted with Ethyl acetate (3 × 50 mL). The combined organic phases were dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo to yield a white solid, which was then suspended in EtOH (35 mL). Concentrated aqueous HCl (5 mL) was added. The mixture was heated at reflux for about 8 h and concentrated to dryness in vacuo. Then the residue was diluted with ethyl acetate (50 mL) and washed with water (20 mL) and brine. The organic phase was dried over anhydrous Na₂SO₄ and concentrated to give a yellow oil. In the last step, the product obtained above was mixed with substitute phenol (6.1 mmol) and K₂CO₃ (1.7 g, 12.2 mmol) in DMF (10 mL) and stirred at 80 °C overnight. After cooling to rt, the reaction solution was diluted with water (30 mL) and extracted with Ethyl acetate (3 × 50 mL). The combined organic layers were washed with brine, dried with Na₂SO₄ and concentrated in vacuo. The residue was purified by flash column chromatography (hexane/ethyl acetate, 2/1, v/v) to provide the final product.

7-(4-Chlorobenzyl)-1-(3-Hydroxypropyl)-3-Methyl-8-(3-(Trifluoromethoxy)Phenoxy)-3,7-Dihydro-1H-Purine-2,6-Dione (HC608).—Yield 20% over 5 steps; white solid; Mp 120–121 °C. ¹H NMR (400 MHz, CDCl₃) δ 7.47 (t, J = 8.1 Hz, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.34 – 7.31 (m, 2H), 7.25 (d, J = 8.6 Hz, 2H), 7.17 (d, J = 8.0 Hz, 1H), 5.44 (s, 2H), 4.22 – 4.20 (m, 2H), 3.58 – 3.54 (m, 3H), 3.45 (s, 3H), 1.93 – 1.92 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 155.19, 153.47, 152.76, 151.52, 149.66, 146.20, 134.45, 134.11, 130.60, 129.76, 129.10, 120.29 (q, J = 258.4 Hz), 118.21, 117.87, 112.98, 102.91, 58.51, 46.57, 37.73, 30.85, 29.90. HRMS (ESI) calcd. for C₂₃H₂₁ClF₃N₄O₅ [M + H]⁺ 525.1147, found: 525.1150.

7-(4-Chlorobenzyl)-8-(3-Chlorophenoxy)-1-(3-Hydroxypropyl)-3-Methyl-3,7-Dihydro-1H-Purine-2,6-Dione (HC-070): Yield 18% over 5 steps; white solid; Mp 131–

132 °C. ¹H NMR (400 MHz, CDCl₃) δ 7.34 – 7.19 (m, 7H), 7.10 (d, *J* = 7.9 Hz, 1H), 5.34 (s, 2H), 4.13 – 4.11 (m, 2H), 3.46 – 3.44 (m, 3H), 3.37 (s, 3H), 1.84 – 1.82 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 155.32, 153.45, 153.07, 151.65, 146.39, 135.19, 134.56, 134.24, 130.66, 129.91, 129.23, 126.38, 120.36, 118.00, 102.99, 58.58, 46.67, 37.81, 30.95, 30.12. HRMS (ESI) calcd. for C₂₂H₂₁Cl₂N₄O₄ [M + H]⁺ 475.0940, found: 475.0934.

8-Bromo-3,7-dihydro-1H-purine-2,6-dione (2).—Under N₂ atmosphere, Xanthine **1** (10.0 g, 66 mmol) and distilled water (65 mL) were added sequentially to a 300 mL sealed tube. Then Br₂ (5.0 mL, 99 mmol, 1.5 equiv) was added slowly with stirring. The sealed tube was capped and heated to 100 °C for 4 hours. After cooling to room temperature, the reaction mixture was filtrated and the solid participate was washed with water and Et₂O. The product was dried in vacuum to give **2** (13.66 g, 90% yield) as a white solid. ¹H (400 MHz, DMSO-*d*₆) δ 11.65 (s, 1H), 10.92 (s, 1H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 154.49, 151.01, 148.77, 124.22, 109.42. HRMS (ESI) calcd. for C₅H₄BrN₄O₂ [M + H]⁺ 230.9512, found: 230.9519.

8-Bromo-7-(4-chlorobenzyl)-3,7-dihydro-1H-purine-2,6-dione (3).—Compound **2** (3.45 g, 15 mmol) and K₂CO₃ (6.21 g, 45 mmol, 3 equiv) were dissolved into DMF (30 mL), and then 1-(bromomethyl)-4-chlorobenzene (3.37g, 16.5 mmol, 1.1 equiv) was added dropwise. The reaction mixture was heated for another 2 hours at 45 °C and quenched by addition of water, extracted with ethyl acetate (3 × 100 mL). The combined organic solution was washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure to afford **3** as a white solid (4.51 g, 85% yield). Mp 300–301 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.40 (d, *J* = 8.0 Hz, 2H), 7.26 (d, *J* = 8.1 Hz, 2H), 5.38 (s, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 156.29, 135.86, 132.25, 129.12, 128.61, 127.03, 108.22, 48.19. HRMS (ESI) calcd. for C₁₂H₈O₂N₄BrClNa [M + Na]⁺ 376.9411, found: 376.9421.

8-Bromo-7-(4-chlorobenzyl)-3-((2-trimethylsilyl)ethoxy)-methyl)-3,7-dihydro-1H-purine-2,6-dione (4).—To a stirred solution of **3** (1.01 g, 2.84 mmol), DBU (432 mg, 2.84 mmol, 1.0 equiv) in DMF (20 mL) was added 2-(Trimethylsilyl)ethoxymethylethoxymethyl chloride (476 mg, 2.84 mmol, 1.0 equiv) at 0 °C. Then the mixture was warmed to rt and heated at 60 °C until TLC demonstrated the reaction was complete. The mixture was quenched with water and extracted with ethyl acetate (3 × 100 mL). The combined organic phases were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (hexane/ethyl acetate, 3:1 to 1:1, v/v) to provide the product as a white solid (892 mg, 65% yield). Mp 148–149 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.47 (s, 1H), 7.37 – 7.30 (m, 4H), 5.47 (s, 4H), 3.72 (d, *J* = 8.4 Hz, 2H), 0.97 (d, *J* = 8.5 Hz, 2H), 0.02 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 153.72, 150.52, 149.84, 134.86, 133.23, 129.76, 129.30, 128.59, 109.19, 71.60, 67.75, 49.88, 18.13, –1.31. HRMS (ESI) calcd. for C₁₈H₂₃BrClN₄O₃Si [M + H]⁺ 485.0406, found: 485.0400.

8-Bromo-7-(4-chlorobenzyl)-1-(3-((tetrahydro-2H-pyran-2-yl)oxy)propyl)-3-((2-(trimethylsilyl)ethoxy)methyl)-3,7-dihydro-1H-purine-2,6-dione (5).—Compound **4** (895 mg, 1.85 mmol), 2-(3-bromopropoxy)tetrahydro-2H-pyran

(453 mg, 2.04 mmol, 1.1 equiv), K₂CO₃ (511 mg, 3.7 mmol, 2 equiv) were mixed in DMF (10 mL) and heated at 60 °C overnight. At completion, the reaction mixture was partitioned between water (50 mL) and ethyl acetate (50 mL). The organic phase were separated, and the aqueous phase was washed with ethyl acetate (2 × 50 mL). The combined organic solution were washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated. The crude was purified by flash column chromatography (hexane/ethyl acetate, 4:1 to 1:1, v/v) to provide the product as a colorless oil (834 mg, 72% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.35 (d, *J* = 8.4 Hz, 2H), 7.30 (d, *J* = 8.5 Hz, 2H), 5.45 – 5.55 (m, 4H), 4.57 (s, 1H), 4.24 – 4.05 (m, 2H), 3.85 – 3.73 (m, 2H), 3.78 – 3.69 (m, 2H), 3.53 – 3.41 (m, 2H), 2.02 – 1.91 (m, 2H), 1.86 – 1.72 (m, 1H), 1.68 – 1.57 (m, 1H), 1.55 – 1.40 (m, 4H), 0.99 (t, *J* = 8.1 Hz, 2H), 0.00 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 170.61, 153.94, 150.65, 147.58, 134.14, 133.31, 129.24, 128.78, 127.55, 108.58, 98.32, 71.73, 67.15, 65.04, 61.74, 60.04, 49.18, 39.10, 30.37, 27.89, 25.28, 20.76, 19.17, 17.68, 14.01, –1.64. HRMS (ESI) calcd. for C₂₆H₃₇BrClN₄O₅Si [M + H]⁺ 627.1400, found: 627.1401.

7-(4-Chlorobenzyl)-1-(3-((tetrahydro-2H-pyran-2-yl)oxy)-propyl)-8-(3-(trifluoromethoxy)phenoxy)-3-((2-(trimethyl-silyl)ethoxy)methyl)-3,7-dihydro-1H-purine-2,6-dione(6).—Compound **5** (834 mg, 1.3333 mmol), 3-(trifluoromethoxy)phenol (284 mg, 1.66 mmol, 1.2 equiv) and K₂CO₃ (367 mg, 2.6666 mmol, 2 equiv) were mixed in DMF (10 mL) and heated at 80 °C overnight. After cooling to rt, the resulting solution was quenched with water and extracted with Ethyl acetate (3 × 100 mL). The combined organic phases were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/ethyl acetate, 4:1 to 3:1, v/v) to yield a colourless oil product (722 mg, 75% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.52 – 7.47 (m, 3H), 7.39 – 7.33 (m, 4H), 7.22 – 7.20 (m, 1H), 5.51 – 5.46 (m, 5H), 4.67 – 4.66 (m, 1H), 4.28 – 4.13 (m, 3H), 3.93 – 3.91 (m, 2H), 3.77 – 3.72 (m, 2H), 3.57 – 3.55 (m, 2H), 2.09 – 2.06 (m, 3H), 1.86 – 1.84 (m, 1H), 1.73 – 1.71 (m, 1H), 1.34 – 1.28 (m, 1H), 0.99 (t, *J* = 7.6 Hz, 2H), 0.00 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 154.63, 153.58, 152.23, 151.05, 149.53, 145.14, 134.22, 130.44, 129.67, 128.96, 120.24 (q, *J* = 258.2 Hz), 117.92, 117.74, 112.77, 102.99, 98.47, 71.94, 67.28, 65.23, 61.85, 60.17, 46.38, 38.99, 30.51, 28.13, 25.40, 19.27, 17.75, 14.05, –1.67, –1.70. HRMS (ESI) calcd. for C₃₃H₄₀ClF₃N₄O₇SiNa [M + Na]⁺ 747.2199, found: 747.2198.

7-(4-Chlorobenzyl)-1-(3-hydroxypropyl)-8-(3-(trifluoro-methoxy)phenoxy)-3,7-dihydro-1H-purine-2,6-dione (7).—To a stirred solution of compound **6** (608 mg, 0.84 mmol) in EtOH (10 mL) was added concentrated hydrochloric acid (1.3 mL) dropwise. Then the mixture was heated under reflux and monitored by TLC until complete consumption of the starting material. After about 8 hours, the reaction solution was cooled and quenched by 10 mL NaHCO₃ (sat.), diluted by water (30 mL) and ethyl acetate (50 mL). The organic phase was separated, and the aqueous phase was washed with ethyl acetate (2 × 50 mL). The combined organic solution was washed with brine, dried over anhydrous Na₂SO₄, filtered and concentrated to dryness in vacuo. The crude was purified by flash column chromatography (hexane/ethyl acetate, 1:2 to 1:3, v/v) to provide the product as a white solid (300 mg, 70% yield). Mp 200–201 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.92 (s,

1H), 7.59 (t, J = 8.3 Hz, 1H), 7.48 – 7.37 (m, 6H), 7.32 – 7.30 (m, 1H), 5.40 (s, 2H), 4.46 (t, J = 5.2 Hz, 1H), 3.97 – 3.79 (m, 2H), 3.45 – 3.41 (m, 2H), 1.88 – 1.38 (m, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 154.70, 153.74, 153.02, 150.58, 148.73 (q, J = 1.8 Hz), 144.66, 135.37, 132.62, 131.36, 129.59, 128.76, 119.96 (q, J = 257.2 Hz), 119.51, 118.42, 113.65, 102.58, 58.84, 45.74, 37.52, 31.11. HRMS (ESI) calcd. for $\text{C}_{22}\text{H}_{18}\text{ClF}_3\text{N}_4\text{O}_5\text{Na}$ [$\text{M} + \text{Na}$] + 533.0810, found: 533.0809.

Radiochemistry

Both of $[^{11}\text{C}]\text{CO}_2$ was produced through the ^{14}N (p, α) ^{11}C nuclear reaction using JSW BC-16/8 cyclotron of Washington University Medical School in Saint Louis. The $[^{11}\text{C}]\text{Methyl iodide}$ ($[^{11}\text{C}]\text{CH}_3\text{I}$) was generated by reduction of $[^{11}\text{C}]\text{CO}_2$ using a nickel catalyst under atmosphere of H_2 at 360 °C, followed by iodination using iodine at 690 °C to prepare $[^{11}\text{C}]\text{CH}_3\text{I}$. Also, the $[^{11}\text{C}]\text{CH}_3\text{I}$ generated was run through the silver triflate (AgOTf) column to produce $[^{11}\text{C}]\text{CH}_3\text{OTf}$ at 228 °C. The resulting $[^{11}\text{C}]\text{CH}_3\text{I}$ or $[^{11}\text{C}]\text{CH}_3\text{OTf}$ was transferred via gas tubing to the reaction vessel in the hot cell.

For radiolabelling reaction condition screening, precursor **7** (0.6–1.0 mg) was dissolved in solvent (0.3 mL), followed by base addition, then the $[^{11}\text{C}]$ reagent was bubbled into the solution until activity peaked. The resulting solution was then heated at 90 °C for 5 minutes during which the vial was shaking occasionally using a long clamp. Then the oil bath was removed and the mixture was cooled and quenched with 1.7 mL HPLC mobile phase. After that, the resulting solution was injected onto the semi preparative HPLC column and the desired radioactivity fraction was collected, diluted with 50 mL water, and loaded onto a C18 Sep-Pak cartridge. The desired $[^{11}\text{C}]\text{HC608}$ solution was extracted with 1.0 mL absolute ethanol elution.

Optimal procedures are given in Table 1, entry 6. $[^{11}\text{C}]\text{CH}_3\text{I}$ was bubbled for 2–3 min into a solution of precursor **7** (0.6–0.8 mg), K_2CO_3 (2.0 mg) in DMF (0.3 mL) at room temperature. Right after the trap of radioactivity reached a plateau, the sealed reaction vessel was heated 5 min using an oil bath at 90 °C. After cooling, the reaction mixture was quenched by 1.7 mL of HPLC mobile phase consisted of 62% acetonitrile and 38% of 0.1 M ammonium formate (v/v, pH 4.5). The diluted reaction mixture was loaded onto a reverse phase semi-prepare C-18 Agilent column (Zorbax SB-C18, 9.2 mm ID $\times$ 250, 5 μm) and then using abovementioned HPLC mobile phase at a flow rate of 4 mL/min, UV detector wave length of 254 nm. The radioactive product was collected from 15 to 16 min into a vial that contained 50 mL of Milli-Q water. The diluted radioactive solution was passed through a plus short C-18 Sep-Pak cartridge (Part No. WAT020515, Waters cooperation, Milford, MA). After using 20 mL of Milli-Q water to rinse the C-18 cartridge, the radioactive product was eluted into a dose vial using 1.0 mL absolute ethanol, and then 9.0 mL solution of 5% Kolliphor HS15 (Sigma-Aldrich) and 85% saline (v/v) was added into the dose vial to formulate an 10% ethanol 10 mL solution prepared for intravenous (IV) injection into animals.

An aliquot of the dose sample (20 μL) was delivered to an analytic HPLC system for authentication by co-injection with cold standard compound **HC608** and determination of the chemical and radiochemical purity, and specific activity. The quality control HPLC

system consisted of an analytical reverse phase HPLC column (Agilent Zorbax SB-C18, 4.6 mm × 250), UV wavelength of 254 nm, mobile phase of CH₃CN/0.1 M HCOONH₄ (v/v, 82/18, pH = 4.5) the flow rate of 1.0 mL/min. At this condition, retention time T_R = 5.4 min. It confirmed that the radioactive product was [¹¹C]HC608 with high specific activity (204–377 GBq/μmol, decay corrected to EOB) and > 99% chemical and radiochemical purity.

Ex vivo biodistribution study in rats

A solution of [¹¹C]HC608 (~3.7 MBq/100 μL, in 10% ethanol, 5% Kolliphor HS15, 85% saline) was injected via tail vein into Sprague Dawley (SD) rats (male; 7 weeks old; 200–220 g). Post injection of the radioactivity, rats were euthanized under anaesthesia at 5, 30, 60 min post-injection (n = 4 per group). Tissues of interests (blood, heart, lung, muscle, fat, pancreas, spleen, kidney, liver, thymus, whole brain) were collected, weighed and counted on an automated Beckman Gamma counter. The uptake of each organ was calculated and expressed as a percentage of the injection dose per gram of wet tissue (%ID/gram).

In vitro autoradiography and H & E staining study

Frozen sections (20 micron) were prepared from adult Sprague Dawley (SD) rat brains (male; 7 weeks old; 200–220 g). The slides were incubated with [¹¹C]HC608 (0.74–1.48 MBq/slide) at room temperature for 30 min with or without the presence of 1 μM of HC070. After incubation, the brain slides were washed 2 min each in the following buffers sequentially: 1X PBS, 10% EtOH in 1X PBS, 30% EtOH in 1X PBS, 1X PBS. Then the rat brain sections were exposed on the Storage Phosphor Screen in an imaging cassette in –20 °C at dark for 3 hr. The distribution of radioactivity was visualized with a Fuji Bio-Imaging Analyzer FLA-7000 (Fuji Photo Film, Tokyo, Japan). Multi Gauge v3.0 software (Fuji Photo Film Co., Tokyo, Japan) was used to quantify the signal (Photo-stimulated luminescence, PSL) from the brain sections. Data were background corrected and expressed as photo-stimulated luminescence signals per square millimeter (PSL/mm²).

After autoradiography, the brain sections were air dried for several minutes before stained with filtered 0.1% Mayers Hematoxylin (Sigma; MHS-16) for 4 minutes in a 50 mL conical tube. Subsequently, the sections were rinsed in cool running double-distilled water for 5 minutes in a Coplin jar. Then the sections were dipped in 0.5% Eosin (1.5g dissolved in 300 mL 95% EtOH) 12 times and distilled water until the eosin stops streaking. Next, sections were dipped in 50% and 70% EtOH 10 times separately before equilibrated in 95% and 100% EtOH for 30 seconds and 1 minute respectively. Finally, coverslips were cleaned with kimwipes and mounted with Cytoseal XYL (Stephens Scientific; cat# 8312–4) before scanning.

Non-human primate (NHP) microPET study

A male adult macaque (7.6 kg) was studied with a microPET Focus 220 scanner (Concorde/CTI/Siemens Microsystems, Knoxville, TN). The animal was scanned under anesthesia (induced with ketamine and secretions reduced with glycopyrrolate) with inhalation 1–2% isoflurane in 40% N₂O and 60% O₂. A 2 h dynamic scan was acquired after a bolus injection 4.0 mL of 310.8 MBq of [¹¹C]HC608 via a venous catheter. For quantitative analyses, PET images were co-registered with MRI images. Three-dimensional

region of interest (the global brain) was identified on the MRI and transformed to the reconstructed PET images to obtain time-activity curves. Activity measures were standardized to body weight and the dose of radioactivity injected to yield SUV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the USA National Institutes of Health (NIH) through the National Institute of Neurological Disorders and Stroke, the National Institute on Aging [NS075527 and NS103988], the American Parkinson Disease Association (APDA), the Greater St. Louis Chapter of the APDA and Barnes Jewish Hospital Foundation. We would like to thank William H. Margenau of Washington University Cyclotron Facilities for ^{11}C radioisotope production. We are also grateful to John Hood, Emily Williams, and Darryl Craig for their assistance with the nonhuman primate microPET studies. We appreciate Drs. Jiwei Gu, Lin Qiu and Joshi Sumit's help in animal study.

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Fig.1.
Structures of lead TRPC5 compounds

Fig.2. [¹¹C]HC608 autoradiography of rat brain sections with radioactive dosage of 0.74, 1.11, and 1.48 MBq/slide respectively under low and high stringent washing conditions. **A.** [¹¹C]HC608 autoradiography of rat brain sections under low stringent washing condition. Tracer dosages were 0.74, 1.11, and 1.48 MBq/slide. **B.** [¹¹C]HC608 autoradiography of rat brain sections under high stringent washing condition. **C.** ARG signals of sections of three rat brain tissue (n = 3, means ±SD) under low stringent washing condition. ARG signal intensity (PSL/mm²) was calculated by dividing the total signal (PSL) with total area (mm²) of the section. **D.** ARG signal of sections of three rat brain tissue (n = 3, means ±SD) under high stringent washing condition. **E.** The total-to-non-specific-binding ratio is the average

signal intensity of three brain sections in each treatment without blocker over that with blocker (**HC070**).

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Fig.3.

The representative sagittal, horizontal, and coronal sections of rat brains. The sections are showing their ARG signal pattern and their anatomic structure of H&E staining from the same brain section. BS, brain stem; Cbm, cerebellum; Ctx, cortex; Hipp, hippocampus; Stri, striatum; Thal, thalamus; SN, substantia nigra.

Fig.4. Summed transverse and coronal PET images (upper panel) and time-activity curve (lower panel) of $[^{11}\text{C}]\text{HC608}$ for microPET study in the whole brain of a male adult cynomolgus macaque. SUV: standardized uptake value.

Scheme 1.

Synthesis of the precursor **7**. Reagents and conditions: (i) Br₂, H₂O, 90%; (ii) 1-(bromomethyl)-4-chlorobenzene, K₂CO₃, DMF, 85%; (iii) (2-(chloromethoxy)ethyl)trimethylsilane, DBU, DMF, 0 °C to 60 °C, 65%; (iv) 2-(3-bromopropoxy)tetrahydro-2H-pyran, K₂CO₃, DMF, 60 °C, 72%; (v) 3-(trifluoromethoxy)phenol, K₂CO₃, DMF, 80 °C, 75%; (vi) HCl (aq), EtOH, reflux, 70%.

Table 1

Optimization of the radiosynthesis condition

Entry	Precursor	[¹¹ C] Source	Base	Yield
1	7 , 1.0 mg	[¹¹ C]CH ₃ I	NaOH(5M), 2 μL	NP ^{a, b}
2	7 , 1.0 mg	[¹¹ C]CH ₃ OTf	/	NP ^{a, b}
3	7 , 1.0 mg	[¹¹ C]CH ₃ OTf	NaOH(5M), 2 μL	NP ^{a, b}
4	7 , 1.0 mg	[¹¹ C]CH ₃ I	NaOH(dry), 5 mg	NP ^{a, b}
5	7 , 1.0 mg	[¹¹ C]CH ₃ I	K ₂ CO ₃ (dry), 5 mg	16% ^c
6	7 , 0.6–0.8 mg	[¹¹ C]CH ₃ I	K ₂ CO ₃ (dry), 2 mg	25 ± 5% ^{c, d}

^aNo yield, meaning no product was detected by HPLC.^bUsing DMSO as solvent.^cRadiochemical yield based on decay corrected yield determined by HPLC.^dReaction was performed for more than 10 batches.

Table 2Biodistribution of [^{11}C]HC608 at 5, 30, and 60 min post injection in SD rats (n = 4)

Tissues	Tracer uptake (%I.D./gram)		
	5 min	30 min	60 min
Blood	0.22±0.05	0.20±0.01	0.16±0.01
Heart	2.06±0.58	1.04±0.05	0.61±0.13
Lung	1.65±0.44	1.13±0.05	0.63±0.13
Muscle	0.22±0.06	0.50±0.02	0.36±0.05
Fat	0.14±0.05	0.72±0.05	0.86±0.23
Pancreas	1.72±0.38	1.49±0.04	0.94±0.20
Spleen	1.26±0.28	0.63±0.03	0.37±0.06
Kidney	2.59±0.50	1.34±0.05	0.82±0.16
Thymus	0.52±0.16	0.63±0.06	0.43±0.07
Liver	4.54±1.03	3.03±0.51	2.22±0.42
Brain	0.51±0.12	0.37±0.01	0.25±0.03