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Synthesis, radiosynthesis and first in vitro evaluation of novel PET-tracers for the dopamine transporter: [^{11}C]IPCIT and [^{18}F]FE@IPCIT

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ABSTRACT

Introduction: Present data indicate that merging beneficial structural elements from previously published DAT-ligands highest DAT affinity, selectivity and a suitable metabolic profile should be achieved. This combination led to the development of IPCIT and FE@IPCIT.
Methods: Precursor synthesis was done starting from cocaine in a six step reaction. *O*-[^{11}C]-methylation was established using [^{11}C]methyl iodide, optimized and subsequently automated. Small scale ^{18}F-fluoroethylation as well as optimization of reaction parameters and automation were performed. Affinity and selectivity of the candidate substances were tested in standard binding experiments on human membranes. Metabolic stability and blood–brain-barrier (BBB) penetration were determined.
Results: Precursor compound, IPCITacid, and reference compounds, IPCIT and FE@IPCIT, were obtained in 4.9%, 12.7% and 4.1% yield, respectively. Automated radiosynthesis of [^{11}C]IPCIT yielded 1.9 ± 0.7 GBq (12.5 ± 4%, corrected for decay). Optimum parameters for ^{18}F-fluoroethylation were 110 °C for 15 min under TBAH catalysis, yielding 67 ± 16% radiochemical incorporation. Affinity was determined as 1.7 ± 0.6 nM for IPCIT, 1.3 ± 0.2 nM for FE@IPCIT and 37 ± 13 nM for the precursor molecule, IPCIT-acid. Results from in vitro and in silico evaluations revealed high stability but also high lipophilicity.
Conclusion: Present data indicate high affinity and stability of both IPCIT and FE@IPCIT. Radiolabelling, optimization of reaction parameters and automation succeeded. On the other hand, data concerning BBB-penetration are not promising.

1. Introduction

The dopamine transporter (DAT) has attracted the attention of neuro-researchers due to its involvement in many neurodegenerative and psychiatric diseases; amongst these Parkinson's disease, attention deficit hyperactivity disorder and schizophrenia.[1–4] As membrane bound monoamine transporter, it facilitates the re-uptake of dopamine into the cytosol and controls the concentration of dopamine in the synaptic cleft. For in vitro and in vivo visualization of DAT, and therefore also for diagnostic investigations of neurodegenerative brain disorders, many SPECT and PET tracers have been developed. These established ligands are mostly based on the nortropane structure of cocaine, such as [^{123}I]β-CIT, [^{11}C]β-CIT, [^{11}C]CFT, [^{18}F]FE@CIT, [^{18}F]FECNT and iodinated nortropane derivatives like [^{123}I]-IPT or [^{125}I]altropane.[5–14] Considering the high concentration of DAT in human brain (B_{max} ~ 200 pmol/g, human putamen), this transporter can be targeted straight-forwardly.[8] Although a variety of radioligands has been described and used in clinical applications, there is still controversy regarding the optimum features for precise quantification of DAT. Low selectivity, slow kinetics, metabolic degradation or problems with blood-brain-barrier (BBB)-penetration put some constraints on their applicability in clinical trials, thus better suitable DAT-PET ligands are still of interest.[15–17]

Halldin and co-workers presented [^{11}C]PE2I and [^{18}F]FE-PE2I as new cocaine congeners and evaluated these in vitro and in vivo.[18–21] These ligands are exhibiting good selectivity and affinity,

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but display certain limitations due to their metabolic fate: two radiometabolites were observed in all brain regions and amongst these one is showing also specific binding to striatum.[22,23] Furthermore, these metabolic transformations show a high inter-individual variability.[22]

Therefore, it was our aim to synthesize novel derivatives combining the para-iodosubstituted phenyl ring, as in the widespread used β-CIT molecule (with good metabolic stability[24]), with an iodopropenyl moiety on the tropane-*N* (derived from the altropane and PE2I structure), as well as with a methyl- or fluoroethyl-ester at the 2β-carbomethoxy function (high affinity and selectivity). This combination led to the development of IPCIT (methyl 8-[(2*E*)-3-iodoprop-2-en-1-yl]-3-(4-iodophenyl)-8-azabicyclo[3.2.1]octane-2-carboxylate and FE@IPCIT (2-fluoroethyl 8-[(2*E*)-3-iodoprop-2-en-1-yl]-3-(4-iodophenyl)-8-azabicyclo[3.2.1]octane-2-carboxylate) (Fig. 1), two DAT tracers with expected high metabolic stability.

In the present work, the objective was to evaluate the affinity and selectivity of the candidate substances and of β-CIT (for comparison) in membrane binding experiments expressing the human monoamine transporters. Moreover, metabolic stability was examined and radiolabelling was optimized with ^{11}C and ^{18}F in small scale experiments and automation was intended.

2. Materials and methods

2.1. Materials

Acetonitrile (ACN) for synthesis of DNA, ⩾99.9% (GC) and ACN (HPLC grade), tetrabutylammonium hydroxide 30-hydrate (TBAH), methanol (MeOH, CHROMASOLV®, for HPLC, ⩾99.9%), ammonium formate, ammonium acetate, acetic acid (⩾99%) and ethanol (absolute) were obtained from Sigma Aldrich (Vienna, Austria). Iodine (sublimated grade for analysis; ACS, Pharm.Eur.) was purchased from Merck (Darmstadt, Germany).

For formulation of the product, 0.9% saline solution from B. Braun (Melsungen, Germany), 3% saline solution (Landesapotheke Salzburg, Austria), sodium dihydrogenphosphate monohydrate and disodiumhydrogenphosphate dihydrate (both from Merck, Darmstadt, Germany) and TWEEN® 80 (polyoxyethylenesorbitan monooleate, Sigma Aldrich, Vienna, Austria) were used. Anion-exchange cartridges (PS-HCO_3) for [^{18}F]fluoride trapping were purchased from Macherey-Nagel (Dueren, Germany). 2-bromoethyl triflate (BET) was synthesized in cooperation with the Department of Drug and Natural Product Synthesis of the University of Vienna (Austria) according to a literature method.[25] Sterile water was purchased from Meditrade Medicare Medizinprodukte (Kufstein, Austria). Phosphate buffer (125 mM) was prepared by dissolving 0.224 g sodium dihydrogenphosphate–monohydrate and 1.935 g disodiumhydrogenphosphate–dihydrate in 100 mL sterile water. For solid phase extraction C18 plus SepPak® cartridges were purchased from Waters (Waters® Associates Milford, USA). Low-protein binding Millex® GS 0.22 μm sterile filters were obtained from Millipore (Bedford, USA). All other chemicals and solvents for the syntheses and radiosyntheses were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (Vienna, Austria) with at least analytical grade and used without further purification.

2.2. Instrumentation

[^{11}C]CO_2 was produced within a GE PETtrace cyclotron (General Electric Medical System, Uppsala, Sweden) by a $^{14}N(p,\alpha)^{11}C$ nuclear reaction under irradiation of a gas target (Aluminium) filled with N_2 (+1% O_2) (Messer Gases, Vienna, Austria). The production of [^{11}C]CH_3I and [^{11}C]CH_3OTf was performed within a Tracerlab™ FX C Pro synthesizer (GE Healthcare, Uppsala, Sweden). [^{18}F]Fluoride was produced within a GE PETtrace cyclotron via $^{18}O(p,n)^{18}F$ reaction (16.5 MeV protons; GE Medical Systems, Uppsala, Sweden). $H_2{}^{18}O$ (HYOX18; >98%) was obtained from Rotem Europe (Leipzig, Germany).

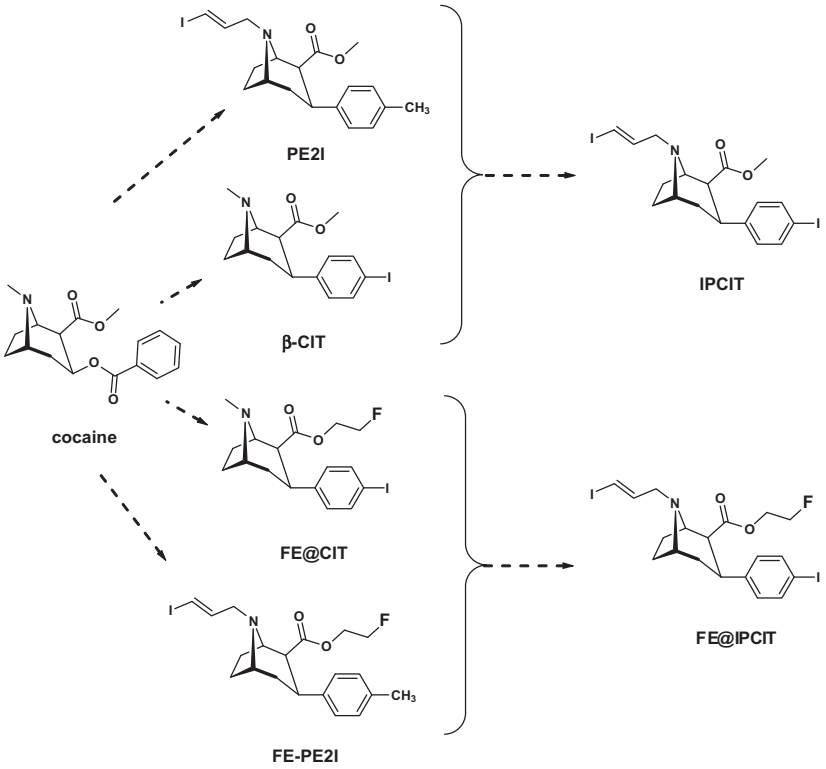


Figure 1. Structures of cocaine and derived DAT ligands.

Evaluation of reaction conditions was performed manually in a lead-shielded hood with small quantities of radioactivity (<1 GBq). After optimization, [^{11}C]IPCIT-synthesis was automated in the Tracerlab™ FX C Pro synthesizer, whereas [^{18}F]FE@IPCIT-synthesis was automated within a Nuclear Interface synthesizer (both GE Medical Systems, Sweden), remotely controlled by a standard laptop with suitable processing software.

Purification of [^{11}C]IPCIT was performed by semi-preparative reversed phase HPLC using the built-in semi-preparative HPLC system equipped with a radioactivity (Bertholdt Technologies, Bad Wildbach, Germany), a UV-detector (Linear Instruments Model 200 Detector UV/vis) and a LaPrep HPLC pump (VWR International, Radnor, USA). A Phenomenex® Gemini, C-18 with TMS endcapping, 10 μm, 250 × 10 mm column (Phenomenex®, Aschaffenburg, Germany) with a mobile phase of MeOH/0.1 M ammonium formate 71/29 v/v containing 1% NEt_3 at a flow rate of 8 mL/min was used for purification.

Analytical HPLC for both tracers was performed on Merck-Hitachi LaChrom HPLC system (L-7100 pump; LaChrom L-7400 UV detector at 254 nm) and a NaI radio-detector (Bertholdt Technologies, Bad Wildbach, Germany) using Raytest software (Raytest, Straubenhardt, Germany). A Chromolith® Performance RP-18e, 5 μm, 100 × 4.6 mm (Merck, Germany) column with a mobile phase consisting of (water/acetic acid 97.5/2.5 v/v; 2.5 g/L ammonium acetate; pH 3.5)/ACN 75/25 v/v at a flow rate of 2 mL/min was used. Osmolality was measured with a Wescor osmometer Vapro® 5600 (Sanova Medical Systems, Vienna, Austria) and pH was measured using a WTW inoLab 740 pH meter (WTW, Weilheim, Germany).

All intermediates and products were analysed spectroscopically via NMR, MS, and HRMS. For NMR analysis, the solvent signal was used as an internal standard which was related to TMS with δ = 7.26 ppm (^{1}H in $CDCl_3$) and δ = 77.0 ppm (^{13}C in $CDCl_3$). NMR: Bruker Avance DPX-200 Spectrometer at 27 °C (200.13 MHz for ^{1}H, 50.32 MHz for ^{13}C); MS: GC/MS-Q95050 GC-17A SHIMADZU; HRMS: Finnigan MAT 8230 (EI, 70 eV) and Finnigan MAT 900 S (ESI, 4 kV, 3 μA CH_3CN/MeOH). NMR analysis (^{1}H and ^{13}C) of intermediates were in full accordance with the literature.

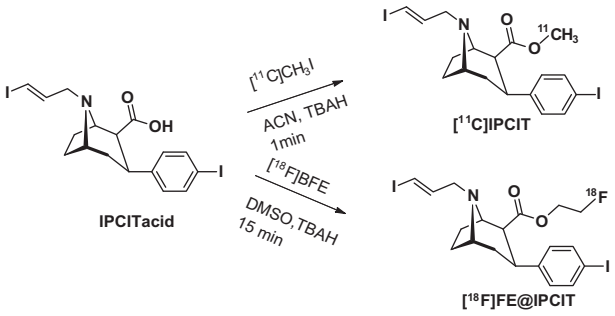
2.3. Methods

2.3.1. Precursor chemistry

Syntheses of precursor and reference compounds were done with some modifications according to previously reported methods (Scheme 1).[26–29] Detailed reaction conditions are given in the Supplementary data.

2.3.2. Radiochemistry

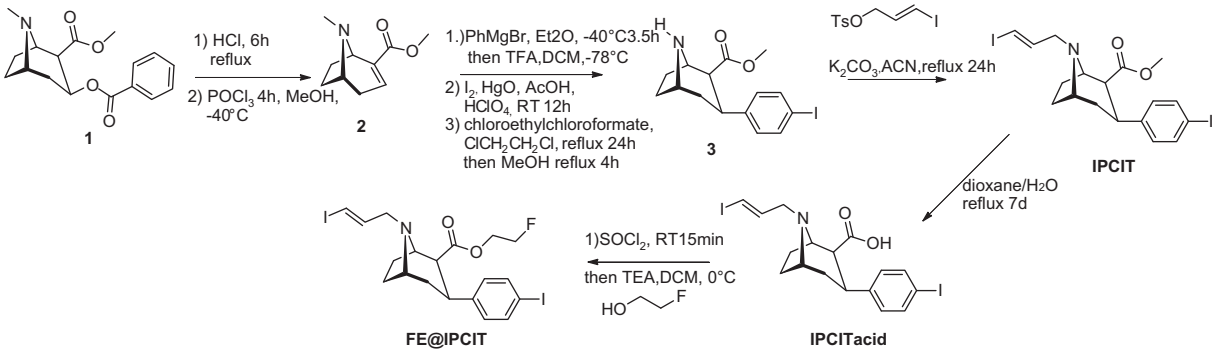
2.3.2.1. Preparation of [^{11}C]IPCIT. *2.3.2.1.1. Production of [^{11}C]CH_3I.* [^{11}C]CO_2 production was stopped as soon as the desired activity (45.7 ± 8 GBq) at currents between 45 and 54 μA was achieved (10–15 min). [^{11}C]CH_3I was produced using a gas phase conversion described by Larsen et al.[30] within the GE Tracerlab™ FX C Pro synthesizer adopting modifications described by Kniess et al.[31] Briefly, [^{11}C]CO_2 was trapped on a molecular sieve (4 Å) within the module and subsequently converted into [^{11}C]CH_4 by a Ni-catalysed reduction with H_2 at 400 °C. The resulting [^{11}C]CH_4 was reacted in a re-circulating process for 4 min with sublimated iodine at 720 °C to give [^{11}C]CH_3I. The produced [^{11}C]CH_3I was trapped on-line on a Porapak® N column and finally released by heating the trap to 190 °C.



Scheme 2. Radiosyntheses of [^{11}C]IPCIT and [^{18}F]FE@IPCIT.

2.3.2.1.2. [^{11}C]IPCIT: small scale experiments and optimization. [^{11}C]methyl iodide was trapped in 500 μL ACN and split for further experiments. All evaluation reactions were, if not stated otherwise, performed in triplicates and executed manually (lead shielded hood, <1 GBq). The impact of reaction time (0.5, 1 and 2 min) and temperature (RT, 50 °C, 75 °C) as well as precursor concentration (0.25, 0.5, 1, 1.5 and 2 mg/mL) were investigated. Final reaction volumes of small-scale reactions were 50–200 μL. In Scheme 2 the radiosyntheses are outlined.

2.3.2.1.3. [^{11}C]IPCIT: automation of synthesis. [^{11}C]-*O*-methylation was automated within a Tracerlab™ FX C Pro syntheziser.[32] Freshly produced [^{11}C]CH_3I was trapped at RT within a glass reactor (2 mL) containing IPCITacid (0.25 mg, 0.48 μmol) and 1 μL of an aqueous TBAH-solution (1 mg/μL) in 250 μL ACN. After stirring for 0.5 min at ambient temperature, the reaction was quenched and diluted by addition of 1 mL water. The crude mixture was transferred to the injection loop and automatically (fluid detector controlled) injected onto the semi-preparative HPLC column. The [^{11}C]IPCIT peak was cut into a bulb, and subsequently diluted with 80 mL water. The aqueous product solution was subjected to solid phase extraction by transfer over a preconditioned (10 mL EtOH, air, 20 mL water, air) C18plus SPE cartridge. After rinsing the C18plus SepPak® with water, the pure product was eluted with 1.5 mL EtOH into a vial containing 100 μL TWEEN®-80 and the



Scheme 1. Syntheses of precursor IPCITacid and reference standards IPCIT and FE@IPCIT.

cartridge and transfer lines were washed with 5 mL 0.9% saline. After sterile filtration (0.22 μm), formulation with further 9 mL 0.9% saline, 1 mL 3% saline and 1 mL 125 mM phosphate buffer was performed under aseptic conditions (laminar air flow hot cell, class A) to avoid microbial contamination.

2.3.2.2. Preparation of [^{18}F]FE@IPCIT. *2.3.2.2.1. Synthesis of 1-bromo-2-[^{18}F]fluoroethane (BFE).* Cyclotron produced [^{18}F]fluoride in $H_2{}^{18}O$ was trapped on a PS-HCO_3 cartridge, and eluted with 0.8 mL of solution A, containing K_2CO_3 (4.5 mg/mL, 33.2 μmol/mL), Kryptofix 2.2.2 (4,7,13,16,21,24-hexaoxa-1,10-diaza-bi-cyclo [8.8.8]hexacosane; 20 mg/mL, 53.2 μmol/mL) in ACN/H2O (70/30 %v/v). Azeotropic drying was performed iteratively by threefold addition of 0.5 mL of ACN. [^{18}F]BFE was synthesized according to Zuhayra et al.[33] A mixture of 30 μL BrEtOTf in 500 μL 1,2-dichlorobenzene (*o*-DCB) was added to the azeotropically dried [^{18}F]fluoride and heated to 100 °C for 10 min. The resulting [^{18}F]BFE was purified by distillation at 100 °C and trapped in 0.5 mL of DMSO at 0 °C.[34] Radiochemical and chemical purity was assessed by analytical HPLC (for conditions see Section 2.1).

2.3.2.2.2. [^{18}F]FE@IPCIT: small scale experiments and optimization. To the resulting [^{18}F]BFE solution in DMSO a solution of precursor IPCITacid and base in DMSO was added. All evaluation reactions were performed manually (shielded hood; starting activity <1 GBq). The influence of reaction time (1, 5, 10 and 15 and 60 min), reaction temperature (RT, 75 °C, 110 °C, 130 °C), base (TBAH, K_2CO_3, Cs_2CO_3, succinic anhydride, NaI, KI, NaH triethylamine and LiOH) and precursor concentration (0.25, 0.5, 1 and 2 mg/mL) was investigated. Finale reaction volumes of small-scale reactions were 50–400 μL. The reaction scheme is presented in Scheme 2.

2.3.2.2.3. [^{18}F]FE@IPCIT: automation of synthesis. Automation of fluoroalkylation was performed within a Nuclear Interface synthesiser. Cyclotron produced [^{18}F]F^- was trapped automatically on an anion exchange cartridge, eluted with 0.8 mL of solution A into the first reactor and iteratively azeotropically dried. Then a solution of 30 μL BET in 500 μL *o*-DCB was added, the vessel sealed and the mixture heated to 100 °C for 10 min. The resulting [^{18}F]BFE was distilled under a smooth He-stream (40 mL/min) into the precooled (0 °C) second reaction vessel containing 0.4 mL DMSO. To this, the precursor (2 mg/mL final concentration), 1 μL of an aqueous TBAH solution (2.4 mg/μL) in 0.2 mL DMSO was added and the sealed reaction vessel heated to 110 °C for 15 min. The crude reaction mixture was cooled to RT and radiochemical conversion checked by analytical HPLC.

2.3.3. Quality control

According to the European Pharmacopoeia, chemical and radiochemical impurities were identified by UV- and radio-HPLC, osmolality and pH were tested with designated equipment. Sterility, absences of endotoxins and residual solvents were determined by routine procedures at the PET Centre of the Vienna General Hospital, Medical University of Vienna. Specific radioactivity was assessed by quantification of the non-radioactive product (HPLC UV channel at 254 nm) and determination of overall radiochemical yield (GBq at end of synthesis).

2.3.4. Statistical analysis

All quantitative data (both in text and figures) are given as arithmetic mean ± standard deviation. A Student *t*-test (two-tailed) was performed for determination of significance; that is, *P* values of <0.05 were considered significant. If not stated otherwise, error bars in figures are representing the standard deviation; if not visible they are within the margin of the symbol.

2.3.5. Membrane binding studies

Affinity of candidate substances was tested in standard DAT-membrane binding experiments.[35,36] A 100 mM NaCl and 50 mM Tris–HCl pH 7.4 was used for the assay. The competitive binding experiments were performed in glass test tubes, filled with 350 μL of the new 'cold' (=non-radioactive) reference compounds, 100 μL of the membrane suspension (in assay buffer; 12.7 μg protein/unit, RBHDATM400UA, Perkin Elmer, Waltham, USA) and 50 μL of a 3 nM ^{3}H-WIN 35,428 (=β-CFT) solution (in assay buffer, 60–87 Ci/mmol, NET1033001MC; Perkin Elmer). For non-specific binding 10 μM GBR 12909 (Sigma–Aldrich, Vienna, Austria) was used; and for total binding (control) only ^{3}H-WIN35,428, buffer and membrane suspension were incubated. After 2 h incubation time at 4 °C, binding was quenched with ice cold buffer, and membrane bound radioactivity was recovered by centrifugation at about 40,000*g* for 15 min. The supernatant was wasted and the pellets were washed two times with 4 mL ice cold assay buffer. After addition of a β-scintillation cocktail (2 mL Ultima GoldTM, biodegradable, Perkin Elmer), the tubes were shaken for 20 min and then counted. Data from the competition plots were analyzed; IC_{50} and K_i values were calculated using GraphPad Prism® 5 software (San Diego, USA). (Arithmetic means of values derived from three different assays, in triplicate for each compound.)

Selectivity of the candidate compounds was tested in NET and SERT membrane studies, similarly to those described for DAT above. NET and SERT expressing membranes were used instead of DAT-membranes (hSERT: 9 μg protein/unit, RBHSTM400UA, Perkin Elmer and hNET: 3 μg protein/unit, RBHNETM400AU, Perkin Elmer). ^{3}H-Nisoxetine*HCl solution (in assay buffer, 70–87 Ci/mmol, NET1084; Perkin Elmer) was as radioligand for the NET assay, ^{3}H-Imipramin*HCl (in assay buffer, NET576; 40–70 Ci/mmol, Perkin Elmer) was used for SERT testing, respectively. IC_{50} and K_i values were obtained in analogy to NET experiments and ratios NET/DAT and SERT/DAT were determined.

2.3.6. Lipophilicity and blood brain barrier penetration

Lipophilicity was tested with HPLC according to Donovan and Pescatore.[37] The candidate substances were injected in a mix of Toluene and Triphenylene (known log*D* and *k*′) in a short polymeric ODP-50 column (20 × 4.0 mm, 5 μm, Shodex®, Showa Denko Europe GmbH, Munich, Germany) using a linear gradient from 10% MeOH/90% 25 mM Phosphate buffer to 100% methanol within 9.4 min at a flow-rate of 1.5 mL/min. As log*D* values are poor predictors for blood brain barrier (BBB) penetration, additional calculation of tPSA (total polar surface area) and IAM chromatography experiments were performed according to Yoon et al. and Tavares et al.;[38,39] tPSA was calculated using ChemBioDraw Ultra 12.0 (CamebridgeSoft, PerkinElmer). For IAM-chromatography, a Reditech IAM.PC.DD2 (Regis Technologies Inc., Morton Grove, USA) column (150 m × 4.6 mm) was used isocratically with 0.01 M phosphate buffer and ACN in different ratios (1 mL/min). Resulting P_m (permeability) and K_m (membrane partition coefficient) were obtained after data analysis and the data were compared with those derived from PE2I and β-CIT as external standards, compounds known to penetrate BBB.

2.3.7. Metabolic stability testing

Metabolic stability was assessed using three different methods. First, plasma stability was determined over a period of 60 min.[40] Therefore, 10 μL of [^{11}C]IPCIT or [^{18}F]FE@IPCIT were incubated in 500 μL pooled human plasma (Innovative Research, Peary Court Novi, Li Heparin, X1693B) at 37 °C using a thermocycler (NB: 2% ethanol in the final incubation solution should not be exceeded, in order to avoid enzymatic side reactions). At the respective time points, an aliquot of the tracer-plasma mixture was quenched with

one volume of an ice-cold methanol/ACN mixture (10/1 v/v). The mixtures were vortexed and centrifuged (4 min, 5 °C, 23,000*g*), and the obtained supernatants were analyzed by radio-HPLC. As result, percentage of metabolized compound per time was obtained.

To investigate the further metabolic fate of [^{11}C]IPCIT and [^{18}F]FE@IPCIT, stability against pooled human liver microsomes (BD Biosciences, Woburn, 20 mg/mL in sucrose) was determined. These microsomes are subcellular fractions containing many drug-metabolizing enzymes (cytochrome P450, flavinmonooxygenases, epoxid hydrolases, etc.). Microsomal incubation was performed similarly to the plasma stability assay described above. Microsome solution in sucrose were pre-incubated for 5 min under physiological conditions (pH 7.4, Phosphate buffer, 37 °C) with a NADPH-generating system (NADP+, glucose-6-phosphate, magnesium-chloride in H_2O and Glucose-6-phosphate dehydrogenase in sodium citrate). Subsequently, 6 μL of the formulated radiotracer were incubated in a final volume of 300 μL microsome solution (NB: 2% ethanol in the final incubation solution should not be exceeded, in order to avoid inhibition of various enzymes like CYPs and UDP-GA).[40] Enzymatic reactions were stopped at the respective time points by quenching with one volume MeOH/ACN (10/1). After vortexing and centrifugation, the supernatants were analyzed by radio-HPLC and the percentage of intact tracer was determined.

Furthermore, stability against carboxylic ester hydrolase (CES) was tested as well. Therefore, 20 μL of a 1/1/1 (v/v/v) mixture of human CES1b, human CES1c and CES2 (each BD Biosciences, Bedford, 5 mg/mL, Cat. No. 453320, 453321, 453322) was prepared and diluted with 270 μL phosphate buffer (pH 7.4). To this 6 μL of the respective radiotracer were added and incubated, stopped and analyzed as described above.

3. Results

3.1. Chemistry

Precursor and reference compounds were synthesized successfully; overall-yields of 4.9% for IPCITacid, 12.7% for IPCIT and 4.1% for FE@IPCIT were achieved.

Briefly, after hydrolysis of cocaine with 6 M HCl, and elimination of the resulting alcohol moiety with $POCl_3$ and MeOH, the anhydroecgonine methyl ester **2** was obtained and purification by distillation yielded 94.3%. After Michael addition of the unsaturated methyl ester with PhMgBr, followed by introduction of the para-iodo substituent at the phenyl ring and N-demethylation with chloroethyl chloroformate in 1,2-dichloroethane, nortropane **3** was obtained in 21.2% yield. N-Alkylation with the (*E*)-3-iodoallyl 4-methylbenzenesulfonate gave the reference compound IPCIT in 63.3% yield. Precursor IPCIT acid was obtained via hydrolysis in dioxane/water in 38.7% yield. Fluoroethylation of IPCITacid resulted in 85.0% of the reference compound FE@IPCIT. Spectroscopic data were in full accordance with the proposed structures (see Supplementary data).

3.2. Radiochemistry

3.2.1. ^{11}C-Radiolabelling

^{11}C-O-methylation evinced no difference whether [^{11}C]methyl iodide or [^{11}C]methyl triflate was used. Using precursor amounts below 0.5 mg/mL, radiochemical incorporation yields (RCIY) were below 30.5%. At 0.5 mg/mL 60% radiochemical incorporation was observed, above 0.5 mg/mL a plateau of 66% RCIY was found (Fig. 2). Base catalysis was in all experiments performed with 1 μL of an aqueous TBAH solution per 500 μL of precursor dissolved in ACN. Increasing temperature from RT to 50 °C or 75 °C appears to play a subordinate role, thus it did not result in a significant increase in radiochemical incorporation. RCIYs for 1 mg/mL precursor concentration at RT ranged from 65.0% for short reaction times (0.5 min at RT) up to 66.4% for longer reaction times (2 min). Hence, small scale experiments revealed optimum conditions of 1 mg/mL precursor concentration, RT, 1 min and TBAH catalysis.

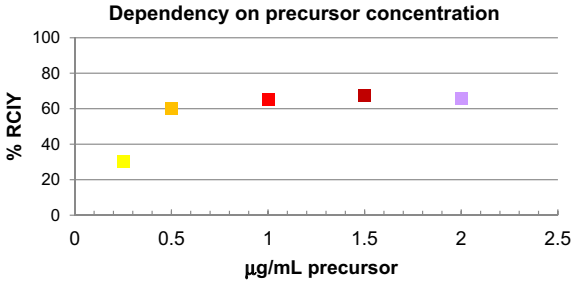


Figure 2. Dependency of RCIY on precursor concentration.

In Table 1, synthesis steps, conversion and yields for large scale preparations are outlined. After preparative HPLC (ret. time [^{11}C]CH_3I = 2.0 min; [^{11}C]ICPIT = 11.5 min) and SPE, the pure product was eluted from the C18plus SepPak® with ethanol and the tubings rinsed with 5 mL saline 0.9%. Sterile filtration was attempted over a 0.22 μm PVDF (polyvinyliden fluoride) or a 0.22 μm MCE (mixed cellulose esters) sterile filter with product solutions with different EtOH concentration. In Figure 3, product passing the sterile filter is depicted. Best conditions were achieved when adding 100 μL TWEEN®-80 to the product solution before sterile filtration.

So far, 5 fully automated radiosyntheses have been performed, yielding 1.9 ± 0.7 GBq [^{11}C]IPCIT (6.4 ± 4%, corr. EOB) within 36 min (for details see Table 1). Specific radioactivities were sufficient with 24 ± 5 GBq/μmol (calculated using an HPLC-based method). Radiochemical and chemical purity were always ⩾98%, osmolality and pH were found to be in a physiological range. GC analysis evinced ACN <5 ppm and methanol <20 ppm. Retention times in the analytical HPLC were 1.05–1.4 min (k' = 0–0.33) for [^{11}C]CH_3I, 2.1–3.0 min (k' = 1.0–1.86) for precursor IPCITacid, and 5.0–5.9 min (k' = 3.76–4.6) for [^{11}C]IPCIT. In Figure 4 a spiked analytical chromatogram of [^{11}C]IPCIT is shown (IPCIT reference standard in 50 μg/mL)

3.2.2. ^{18}F-Radiolabelling

[^{18}F]BFE was obtained in sufficient yields (26.4 ± 6%) and high purity (⩾95%) after distillation. ^{18}F-Fluoroethylation was performed in small scale experiments investigating the influence of base, reaction temperature and precursor concentration. Regarding the reaction temperature, very low RCIYs (2.9%) were obtained for reactions at RT, whereas high RCIYs were achieved at 110 °C (55.1–78.1%) after 15 min. Elevation of temperature to 130 °C did not result in further increase in radiochemical incorporation. Elongation of reaction time did not affect RCIY. Using precursor amounts of more than 1 mg/mL did not show a beneficial effect either. No conversion of [^{18}F]BFE to [^{18}F]FE@IPCIT was observed for reactions below 0.5 mg/mL. In Table 2 the effects of base catalysis are summarized. Advantages of addition of NaI or KI could not be confirmed for this reaction. Hence, optimum conditions were obtained using 1 mg/mL IPCITacid at 100 °C with TBAH catalysis (1 μL, aqueous solution).

Although the optimum conditions were used for automation in larger scale, unexpectedly poor radiochemical incorporation yields (below 6%) were achieved.

Table 1
Fully automated preparation of [^{11}C]IPCIT with $n \geqslant 5$ (∗at end of synthesis)

$n \geqslant 5$	GBq	% of initial activity (corr. for decay)	Δt to EOB (min)
[^{11}C]CO_2 targetactivity	45.7 ± 8.7	100	0
[^{11}C]CH_3I activity trapped in reactor	19.8 ± 2.2	71.7 ± 9.1	14 ± 1
Residual in reactor after transfer to HPLC	1.0 ± 0.6	3.8 ± 2.2	16 ± 1
Residual in HPLC injection loop waste	1.1 ± 1.0	4.2 ± 4.5	16 ± 1
Collected [^{11}C]IPCIT before sterile filtration	2.1 ± 0.4	13.8 ± 1.9	33 ± 1
[^{11}C]IPCIT final product yield	1.9 ± 0.7	12.5 ± 4.4	36 ± 2
Specific activity	24 ± 5		

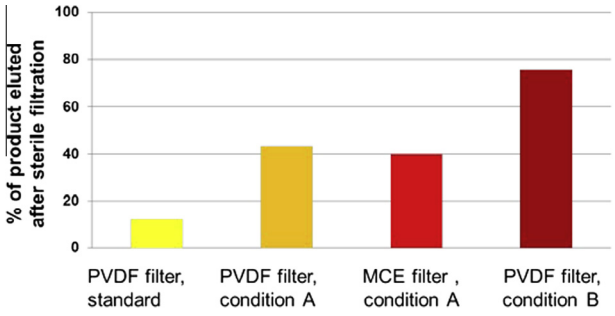


Figure 3. Percentage of product passing the 0.22 μm sterile filter using different conditions for product transfer over sterile filter: *standard:* 9 mL 0.9% saline, 1 mL 3% saline, 1 mL 125 mM phosphate buffer; 1.5 mL EtOH; *condition A*: 5 mL 0.9% saline, 1.5 mL EtOH; *condition B*: 5 mL 0.9% saline, 1.5 mL EtOH, 100 μL Tween® 80).

The radiochemical and chemical purity was assessed via analytical HPLC (see Section 2.1), the retention times were: [^{18}F]F$^-$: 1.6-1.8 min (k' = 0–0.1), IPCITacid: 2.05–2.2 min (k' = 0.28–0.38), [^{18}F]BFE: 3.8–4.2 min (k' = 1.37-1.63) and [^{18}F]FE@IPCIT: 5.0-6.5 min (k' = 2.1-3.06). In Figure 4 an exemplary analytical HPLC chromatogram is shown (co-injection with FE@IPCIT reference standard 50 μg/mL)

3.3. Affinity and selectivity testing, lipophilicity and blood brain barrier penetration

Optimum conditions for DAT-affinity testing were found to be incubation at 25 °C in a buffer containing 100 mM NaCl and 50 mM TRIS*HCl at pH 7.4. K_i values of reference compounds were determined as 37 ± 13 nM for precursor IPCITacid, 1.7 ± 0.6 nM for IPCIT and 1.3 ± 0.2 nM for FE@IPCIT ($n \geqslant 5$ triplicates). For testing of selectivity of our candidate compounds, affinity towards NET and SERT was determined similarly, revealing an 11-fold selectivity for IPCIT and a 115-fold selectivity for FE@ICPIT towards DAT as compared to NET. Selectivity towards SERT was found to be poor for IPCIT, but tolerable for FE@IPCIT (SERT/DAT: 1.8-fold).

Log$D$ was determined as 0.87 for precursor IPCITacid, 5.29 for IPCIT and 5.39 for FE@IPCIT. Regarding BBB-penetration a tPSA value of 29.54 was calculated both for IPCIT and FE@IPCIT. IAM-chromatography experiments revealed permeability (P_m) values of 0.04 for IPCITacid, 7.74 for IPCIT and 8.94 for FE@IPCIT. For comparison P_m was also determined for PE2I (3.15) and β-CIT (0.31), two compounds known to penetrate the BBB. In Table 3, all tested preclinical parameters including K_i, P_m, PSA and log$D$ are outlined.

3.4. Metabolic stability testing

The aim of this molecular design was to obtain a DAT PET tracer with both high affinity and metabolic stability. Therefore, three different metabolizing systems were used to determine the metabolic fate of our novel candidate tracers over a period of 1 h, representative for the PET measurement duration. In Figure 5, an overview of the degradation is given. Hereby, no metabolism was observed in human plasma, thus 100% of the tracers were intact after 1 h of incubation. Using human liver microsomes, a significant metabolic degradation was observed. After 1 h of incubation 46.4 ± 0.6% of [^{11}C]IPCIT and 71.6 ± 2.7% [^{18}F]FE@IPCIT were found to be intact. After incubation with CES, 92% of the candidate tracers were found to be intact after 60 min.

4. Discussion

The rationale of this work was to combine the structural advantages of β-CIT and PE2I, aiming at DAT-ligands with high affinity, stability and selectivity. Synthesis of precursor and reference compounds was achieved successfully. In preliminary preclinical examinations, affinity was found to be high; also selectivity was sufficient due to the high abundance of DAT in human brain as compared to other monoamine transporters.

Optimized O-^{11}C-methylation was leading to good radiochemical incorporation yields with low amounts of precursor at ambient temperature. Furthermore, also purification of crude mixture via preparative HPLC and SPE succeeded. Sterile filtration was optimized using 100 μL TWEEN®-80. Automation of ^{11}C-radiosynthesis yielded 1.9 ± 0.7 GBq [^{11}C]IPCIT within 36 min, enabling large scale preparations (starting activities: 40–50 GBq [^{11}C]CO_2) under maximum radiation safety compliance.

Specific activities were moderate with 24 ± 5 GBq/μmol, due to difficulties with [^{12}C]CO_2 impurities in the target gas (nota bene: this was observed for all ^{11}C-labelled tracers at that time, but was resolved in the meantime). Quality control of [^{11}C]IPCIT showed high purity (<99%) of all batches of the formulated product observing the European Pharmacopoeia and therefore allowing for further preclinical testing.

Preparation of [^{18}F]BFE was done successfully starting from [^{18}F]F$^-$ and 2-bromoethyltriflate in o-DCB and purified by distillation. Trace amounts of o-DCB turned out to hamper the reaction also in very small amounts, when present in the reaction mixture; therefore a rather smooth He-stream during distillation (⩽40 mL/min) was crucial. Fluoroalkylation of IPCITacid with [^{18}F]BFE was accomplished in small scale experiments in high radiochemical yields (up to 66.6 ± 16%) using 1 μL of an aqueous TBAH solution. Other alkaline compounds were evaluated as catalyst, amongst these only Cs_2CO_3 showed similar catalytic activity as TBAH. The addition of small amounts of KI or NaI to the reaction mixture was reported to be beneficial, however this was not observed for this specific ^{18}F-fluoroalkylation reaction. On the contrary, lower RCIYs were observed when adding KI or NaI.[24] Increasing the reaction temperature also did not result in further increase in radiochemical incorporation, thus maximum conversion was observed at 110 °C for 15 min. Automation using optimum parameters did not lead to RCIYs higher than 6%.

In a first preclinical evaluation, we found high affinity towards DAT for both candidate compounds. The DAT-affinity of IPCIT is 10-fold higher than the affinity displayed by PE2I towards DAT. A more than 13-fold affinity was determined for FE@IPCIT at DAT as compared to PE2I. Unfortunately, selectivity DAT/SERT is poor for both ligands. Thus, the hypothesis of this molecular design, did not lead to ligands with higher selectivity. Nevertheless, the selectivity is tolerable, as also known from other DAT radioligands in clinical use (e.g., [^{123}I]FP-CIT, DATScan®).

Metabolic stability was expected to be high due to the favorable combination of structural elements from PE2I and β-CIT (no benzylic oxidation possible), but displayed to be high only against human plasma and human carboxyl esterase, and moderate against human liver microsomes. Here, only polar metabolites were

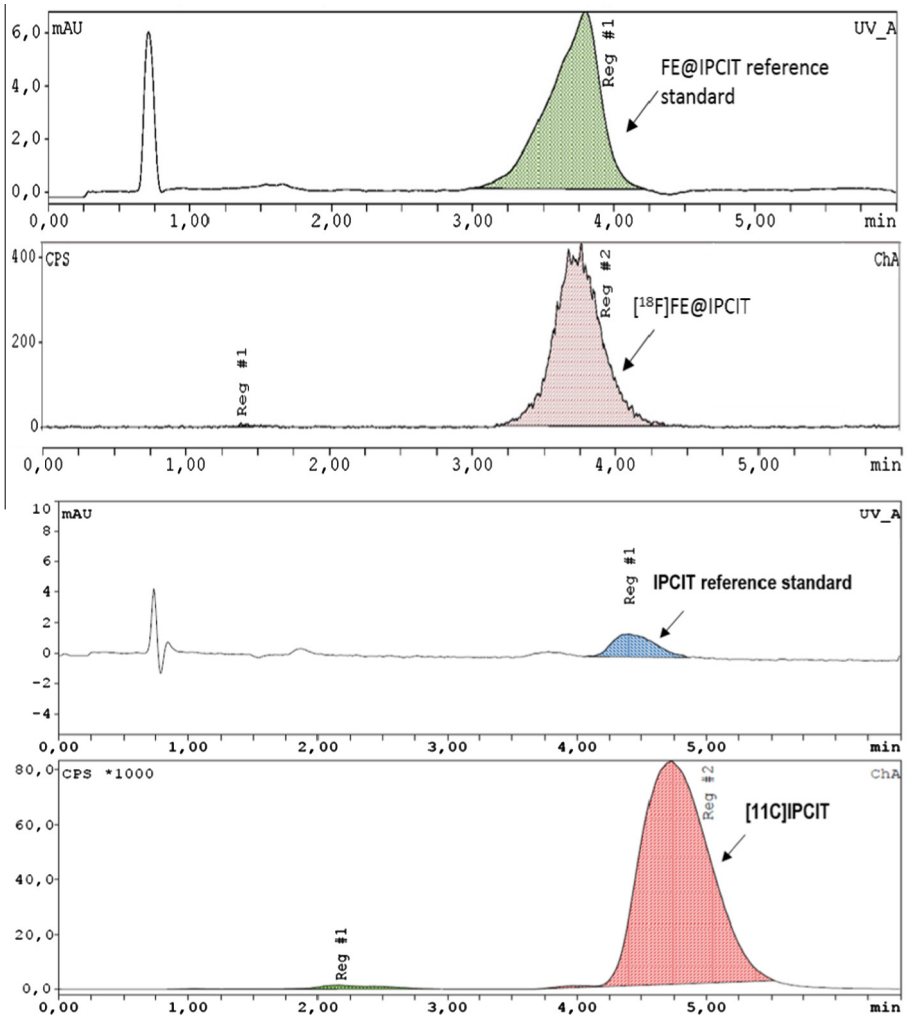


Figure 4. Co-injected analytical HPLC chromatograms of [^{11}C]IPCIT and IPCIT reference standard and [^{18}F]FE@IPCIT and FE@IPCIT reference standard.

Table 2
Effects of different bases on RCI of ^{18}F-fluoroethylation of IPCITacid (110 °C, 15 min, 1 mg/mL IPCITacid)

FE@IPCIT base catalysis	TBAH	Cs_2CO_3	LiOH	Na_2 succinate	KI + Cs_2CO_3	NEt_3	KOH, NaOH, KI, NaH, TRIS, NaI or KI + NaH
% RCI	78	75	70	31	21	2	0

Table 3
Overview on tested preclinical parameters of DAT ligands

					K_i (nM)		
	log*D*	tPSA[a]	K_M	P_m	DAT	NET	SERT
IPCITacid	0.87	40.54	21.26	0.04	36.99 ± 13	785 ± 179	71.4 ± 25.5
IPCIT	5.29	29.54	4157.34	7.74	1.72 ± 0.6	20 ± 10	1.5 ± 0.4
FE@IPCIT	5.39	29.54	5086.15	8.94	1.33 ± 0.2	153 ± 53	2.4 ± 1
PE2I	4.71[a]	29.54	1340.99	3.15	17 ± 7[42]	>1000[42]	500 ± 30[42]
b-CIT	3.69[a]	29.54	120.43	0.31	6.34 ± 1.7[22]	32.77 ± 13.41[22]	29.17 ± 6.4[22]

[a] Calculated with ChemBioDraw Ultra 12).

formed. Thus, no critical interaction on the target sites in vivo can be expected. Overall, metabolic stability tests indicate higher stability than for previously published DAT ligands.[7,13,16,20]

Considering a potential in vivo application of [^{11}C]IPCIT or [^{18}F]FE@IPCIT as DAT-PET tracer, also BBB-penetration was examined. Therefore, log*D* was measured and tPSA calculated. According to Yoon et al. a tPSA value below 60 seems to predict possible BBB-penetration.[38] We found a tPSA value of 29.54 for both IPCIT and FE@IPCIT. Nevertheless, being aware of the high log*D* value of >5 for both candidate ligands, and a measured high P_m value (>8) in IAM experiments, crossing of BBB and high specific brain uptake might be doubted.

Since previously published DAT ligands also showed high lipophilicity (e.g., [^{123}I]IPT[41]) and lack a testing of IAM chromatography and tPSA values, a possible BBB penetration cannot be totally excluded for our candidate DAT tracers although the found values

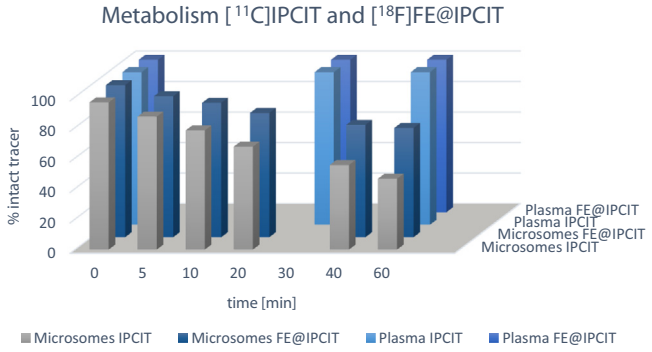


Figure 5. Metabolic stability of [^{11}C]IPCIT and [^{18}F]FE@IPCIT against human CES, plasma and microsomes.

suggest otherwise. Further examinations of the candidate ligands in biodistribution studies could be performed to clarify whether a significant brain uptake is possible or not. However, in respect to the 'three Rs-principle' (reduction, refinement, replacement)[42] we think that such additional animal (in vivo and ex vivo) experiments are not justified enough. Therefore, we think that data concerning BBB-penetration from in vitro and in silico experiments are representative and allow to judge the suitability of our molecules to be used in further studies.

Consequently, we acknowledge that merging two structurally beneficial elements did, in this case, not result in an improved DAT-PET ligand for in vivo application.

5. Conclusion

Synthesis and radiosyntheses of the candidate compounds were performed successfully. Membrane binding experiments revealed high affinity for both methylated and fluoroethylated compounds; selectivity and metabolic stability turned out tolerable. Both candidate compounds displayed high log$D$ and P_m values, making BBB-penetration questionable.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.046.

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