Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 28 January 2014, Revised 17 March 2014, Accepted 19 March 2014 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.3199

[18F]DPA-C5yne, a novel fluorine-18-labelled analogue of DPA-714: radiosynthesis and preliminary evaluation as a radiotracer for imaging neuroinflammation with PET

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DPA-C5yne, the lead compound of a novel series of DPA-714 derivatives in which the fluoroethoxy chain linked to the phenylpyrazolopyrimidine scaffold has been replaced by a fluoroalkyn-1-yl moiety, is a high affinity (K_i: 0.35 nM) and selective ligand targeting the translocator protein 18 kDa. In the present work, DPA-C5yne was labelled with no-carrier-added [18F] fluoride based on a one-step tosyloxy-for-fluorine nucleophilic substitution reaction, purified by cartridge and HPLC, and formulated as an *i.v.* injectable solution using a TRACERLab FX N Pro synthesizer. Typically, 4.3–5.2 GBq of [18F]DPA-C5yne, ready-to-use, chemically and radiochemically pure (> 95%), was obtained with specific radioactivities ranging from 55 to 110 GBq/μmol within 50–60 min, starting from a 30 GBq [18F]fluoride batch (14–17%). LogP and LogD of [18F]DPA-C5yne were measured using the shake-flask method and values of 2.39 and 2.51 were found, respectively. Autoradiography studies performed on slices of ((R,S)-α-amino-3-hydroxy-5-methyl-4-isoxazolopropionique (AMPA)-lesioned rat brains showed a high target-to-background ratio (1.9 ± 0.3). Selectivity and specificity of the binding for the translocator protein was demonstrated using DPA-C5yne (unlabelled), PK11195 and Flumazenil (central benzodiazepine receptor ligand) as competitors. Furthermore, DPA-C5yne proved to be stable in plasma at 37°C for at least 90 min.

Keywords: fluorine-18; radiosynthesis; DPA-C5yne; DPA-714; TSPO 18 kDa; PBR

Introduction

Overexpression of the translocator protein (TSPO) 18 kDa (formerly known as the peripheral benzodiazepine receptor (PBR)[1]) in the brain, during microglial cell activation in response to cerebral insults, has attracted considerable attention for the development of positron emission tomography (PET) radioligands as imaging markers of neuroinflammation.[2,3]

While several radioligands are still underdevelopment,[4–9] the pyrazolo[1,5-*a*]pyrimidine-acetamide [18F]DPA-714[10–12] is currently considered as a major challenger[13–23] of the reference ligand, the isoquinoline-carboxamide [11C]PK11195,[24,25] together with the phenoxypyridin-3-yl-acetamide [11C]PBR28[26–32] and its closely related fluorinated analogue [18F]FEPPA.[33,34] However, the use of radioligands belonging to the latter class faces difficulties today in human PET imaging because of differences between individuals linked to the variable expression of two binding sites (a low and a high affinity) for the TSPO, encoded by a single polymorphism (rs6971) at the gene level.[35–37] Subjects may thus be classified as high-affinity binders or low-affinity binders (when one single binding site for the TSPO is expressed with either high or low affinity, but may also be classified as mixed affinity binders when both sites are expressed approximately equally. Differences in affinity are higher for [11C]PBR28 (50-fold), whereas pyrazolo[1,5-*a*]pyrimidine-acetamides show lower differences (only fourfold for [11C]DPA-713).[36]

On the other hand, recent in-house studies have demonstrated that [18F]DPA-714 is extensively metabolized *in vivo* in both rodents and non-human primates.[38] One metabolic pathway, which was demonstrated *in vitro* and was also suggested to occur *in vivo*, results from *O*-dealkylation that leads to the formation of fluorine-18-labelled small metabolites such as [18F] fluoroacetaldehyde, or its oxidation product [18F]fluoroacetic acid.

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The main issue is that [^{18}F]fluoroacetate is known to cross the blood brain barrier at a certain level[39–41] and may thus confound the brain PET signal. Moreover, [^{18}F]fluoroacetate may be converted to [^{18}F]fluoride ion,[42–44] which bind avidly to the bones, including the skull.[45] This accumulation of radioactivity in the skull is also problematic as far as brain imaging is concerned because it interferes with the quantification of the radiotracer due to the partial volume effect.[45]

Consequently, structural modifications of the DPA-714/DPA-713 chemotype that could prevent *O*-dealkylation and thus avoid the formation of [^{18}F]fluoroacetate and [^{18}F]fluoride ions were explored. While keeping the scaffold of DPA-714, new analogues have recently been synthesized, in which the oxygen atom bridging the phenyl ring and the fluoroalkyl chain is replaced with a carbon atom. Of particular interest is a novel subclass of compounds featuring an alkyne bond and a short alkane spacer linking the phenylpyrazolopyrimidine scaffold and the fluorine atom.[46] This series includes four analogues of DPA-714 with a side-chain length ranging from three to six carbon atoms, which all exhibit high affinity and selectivity towards the TSPO. Within this series, DPA-C5yne (**1**, Figure 1) exhibited the highest affinity for the TSPO with a K_i value of 0.35 nM (as reference, the Ki measured for DPA-714 in the same assay was 0.91 nM). No affinity for the central benzodiazepine receptor (CBR) was observed ($K_i > 1\ \mu M$).[46] Furthermore, preliminary *in vitro* metabolism evaluation of this series using rat microsomal incubations and liquid chromatography–mass spectrometry (LC–MS) analyses showed the absence of defluorinated metabolites.[46]

Based on these data, DPA-C5yne (**1**) was selected for labelling with the short-lived positron-emitter fluorine-18 ($T_{1/2}$: 109.8 min). The present manuscript briefly describes the preparation of the tosyloxy derivative as precursor for labelling and then focuses on the radiolabelling with fluorine-18 of **1** on a TRACERLab FX N Pro module, the measurement of its lipophilicity (LogP/LogD) based on the shake-flask method and its preliminary evaluation by autoradiography on brain slices of our in-house-developed rat model of acute local neuroinflammation.

Results and discussion

Chemistry

The preparation of DPA-C5yne (**1**, *N*,*N*-diethyl-2-(2-(4-(3-fluoropent-1-yn-1-yl)phenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide) as reference compound and the tosylate **2** (6-(4-(3-(2-(diethylamino)-2-oxoethyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-2-yl)phenyl)pent-4-yn-1-yl) 4-methylbenzenesulfonate) as precursor for labelling with fluorine-18 is illustrated in Scheme 1. Briefly, both compounds were synthesized in a single step from the alkynol **3**, the latter being prepared in five chemical steps from commercially available methyl 4-iodobenzoate.[46] Then, fluorodeoxygenation of **3** was performed using Deoxofluor® (Sigma-Aldrich, Saint-Quentin Fallavier, France) in dichloromethane at room temperature for 3 days and afforded the corresponding fluorinated compound, DPA-C5yne (**1**), in 24% yield. The tosylate **2** was also prepared from the alkynol **3**, by treatment with 4-toluenesulfonic anhydride in dichloromethane at room temperature for 2–3 days, and obtained in 54% yield.

Radiochemistry

DPA-C5yne (**1**) was labelled with fluorine-18 from the tosyloxy derivative **2**, purified by HPLC and formulated as an *i.v.* injectable solution by implementing the one-step radiochemical process outlined in Scheme 2. The whole procedure was performed using a commercially available TRACERLab FX N Pro synthesizer (GE Medical Systems, Germany). The positioning of all reagents, solvents and consumables dedicated to the preparation of [^{18}F]DPA-C5yne ([^{18}F]-**1**) on the TRACERLab FX N Pro is illustrated on the control screen copy displayed in Figure 2. Further details are given in the Experimental Section.

No-carrier-added K[^{18}F]F-Kryptofix®222 (Sigma-Aldrich, Saint-Quentin Fallavier, France) complex[47,48] was prepared from cyclotron-produced [^{18}F]fluoride, K_2CO_3, Kryptofix®222, acetonitrile and water using a step-by-step sequence (time list) developed by GEMS and implemented on the synthesizer at its reception on site. The sequence includes the trapping of [^{18}F]fluoride anions on a dedicated QMA cartridge, their release using a H_2O/ CH_3CN solution containing both K_2CO_3 and Kryptofix®222 and a two-step concentration-to-dryness process in a vessel (reactor) made from glassy carbon. K[^{18}F]F-Kryptofix®222 was obtained in 18 min and used without any additional drying sequence.

Radiofluorination was then performed by dilution of the aforementioned reagent using a DMSO solution (700 µL) containing 7.0–8.7 µmoles (4.0–5.0 mg) of the tosylate **2**, followed by stirring and heating. Initially, the conditions used were those optimized for the preparation of [^{18}F]DPA-714, for example, 165°C for 5 min.[12] Using the latter, [^{18}F]DPA-C5yne ([^{18}F]-**1**) was formed and could be isolated after cartridge/HPLC purifications (see in the succeeding texts). However, overall

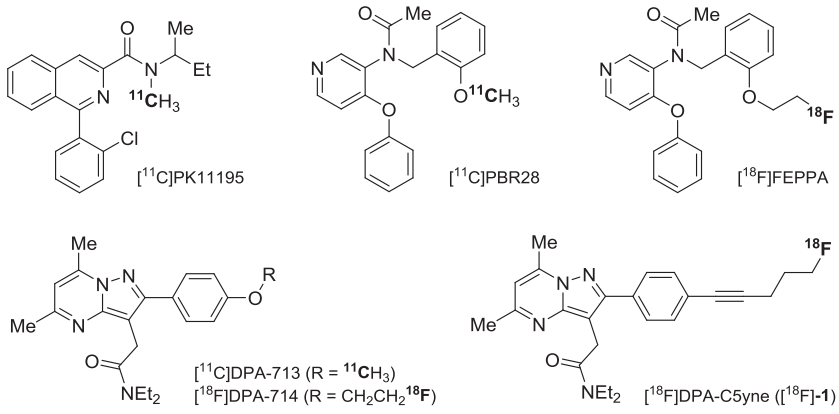
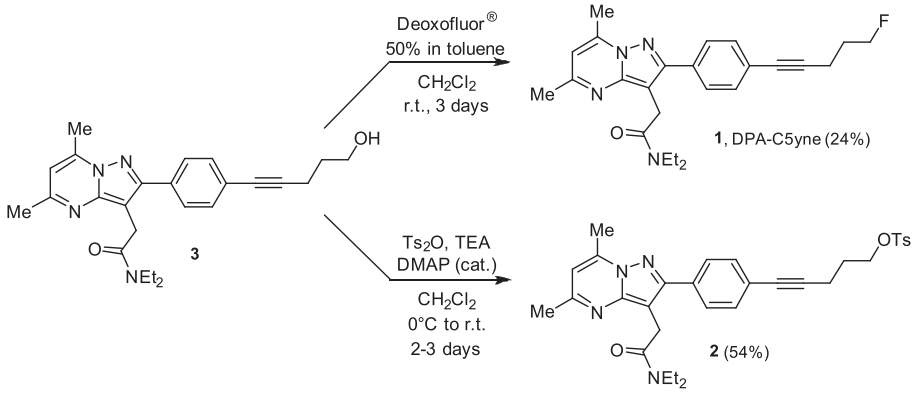
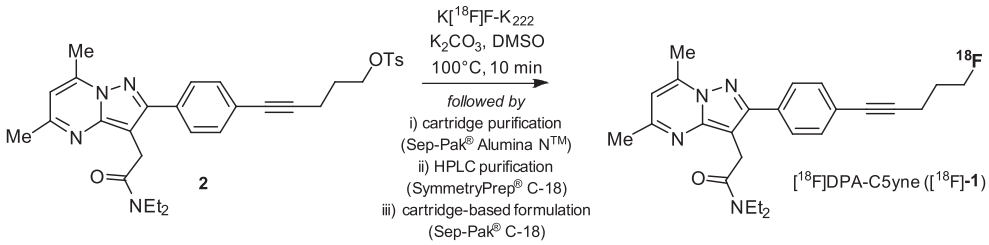


Figure 1. The TSPO radioligands [^{11}C]PK11195, [^{11}C]PBR28, [^{18}F]FEPPA, [^{11}C]DPA-713, [^{18}F]DPA-714 and [^{18}F]DPA-C5yne ([^{18}F]-**1**, present work).



Scheme 1. Synthesis of DPA-C5yne (**1**, reference compound) and its tosylated analogue (**2**) as labelling precursor.



Scheme 2. One-step radiosynthesis, purification and formulation of [^{18}F]DPA-C5yne ([^{18}F]-**1**).

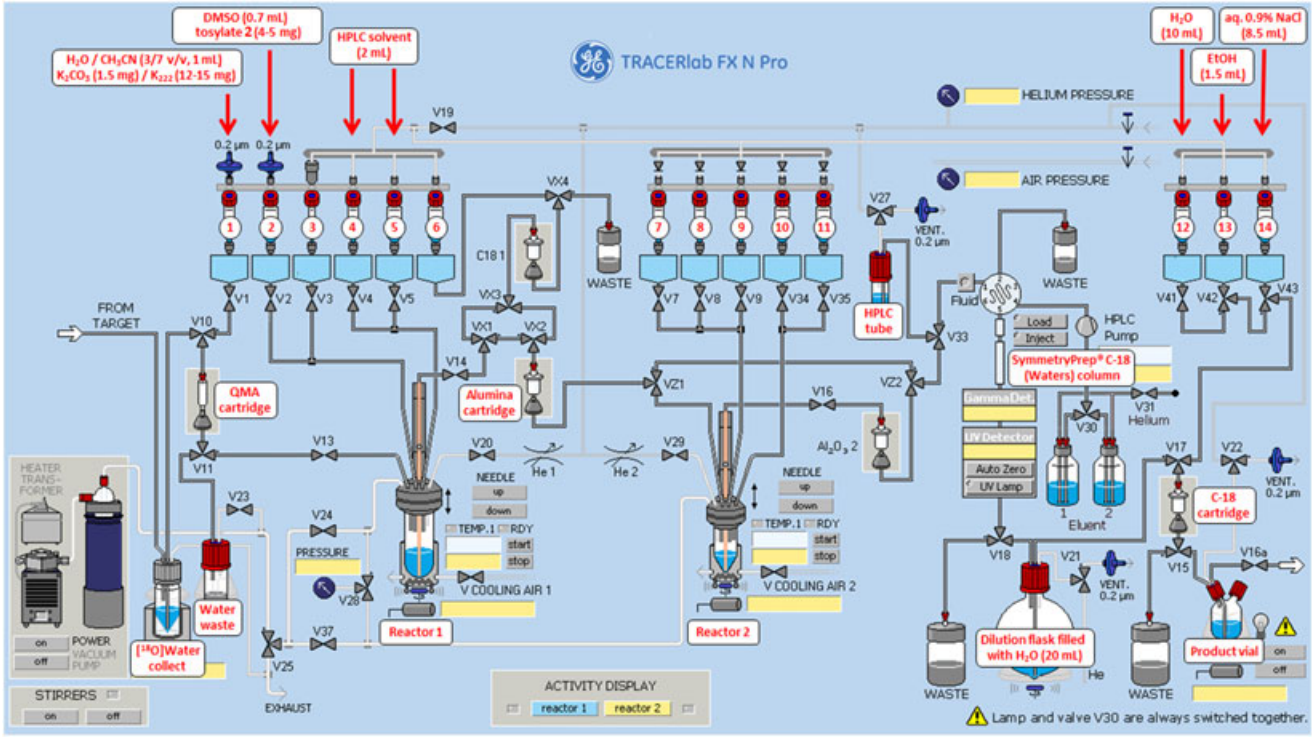


Figure 2. Description of the TRACERLab FX N Pro synthesizer used for the preparation of [^{18}F]DPA-C5yne ([^{18}F]-**1**) and positioning of all required reagents, solvents and consumables.

yields were rather low and not reproducible. Moreover, high chemical and radiochemical purities were not systematically reached based on Quality Control (QC) analysis. These behaviours were not observed during the preparation of [^{18}F]DPA-714. Decreasing the reaction temperature together with slightly extending the reaction time (100°C/10 min) provided [^{18}F]DPA-C5yne ([^{18}F]-**1**) in higher and more reproducible yields without any QC issues.

The crude reaction mixture was then cooled down to 50°C before being diluted with the HPLC solvent used for the

purification and then passed through an Al_2O_3 cartridge (Sep-Pak® Alumina N™ cartridge, Waters (Guyancourt, France)) in order to remove (trap) unreacted [^{18}F]fluoride. The left-over activity in the reactor was then rinsed once with a second portion of HPLC-solvent and passed through the cartridge too. The combined fractions were then purified by HPLC on a semipreparative SymmetryPrep® C-18 column (Waters), using a mixture of CH_3CN, H_2O and Trifluoroacetic acid (TFA) as the eluent (HPLC D, see Experimental Section). Using the latter conditions, [^{18}F]-**1** (t_R: 9–10 min) could be obtained with a > 95% chemical and radiochemical purity and was completely separated from the remaining tosylate **2** (t_R: 15–17 min). Removal of the HPLC solvents and formulation of [^{18}F]DPA-C5yne ([^{18}F]-**1**) as an *i.v.* injectable solution (0.9% aq NaCl containing EtOH) were performed using a reverse phase cartridge (Sep-Pak®Plus C18 cartridge). The whole process, including radiofluorination, cartridge and HPLC purifications, as well as formulation, lasted between 37 and 42 min.

Typically, 4.3–5.2 GBq of [^{18}F]DPA-C5yne ([^{18}F]-**1**) ready-to-use was obtained with specific radioactivities ranging from 55 to 110 GBq/μmol within 50–60 min (HPLC purification and Sep-Pak®-based formulation included), starting from a 30 GBq [^{18}F]fluoride batch. Overall, non-decay corrected, isolated yields were 14–17% (20–25% decay corrected).

Quality controls of [^{18}F]DPA-C5yne ([^{18}F]-**1**) were performed on an aliquot of the ready for *i.v.* injection batch. The prepared radiotracer solution was clear and colourless with a pH between 6 and 7. As demonstrated by analytical-HPLC control (HPLC E, see Experimental Section), the radiotracer preparation was found to be >95% chemically and radiochemically pure (**1**, t_R: 2.20 min). The preparation was also shown to be free of the non-radioactive precursor, the tosylate **2** (t_R: 3.51 min), and was chemically and radiochemically stable for at least 120 min.

Lipophilicities

LogP (*n*-octanol/water partition coefficient) and LogD (*n*-octanol/buffer pH 7.4 partition coefficient) of [^{18}F]DPA-C5yne ([^{18}F]-**1**) were measured using the shake-flask method, and values of 2.39 and 2.51 were found, respectively. The calculated LogP (cLogP) was determined using ChemBioDraw Ultra software, and a value of 4.28 was found. These values were all slightly higher than the ones previously obtained for [^{18}F]DPA-714 (LogP: 1.66, LogD: 1.74 and cLogP: 3.33) but remained in the recommended range for a radiotracer dedicated to brain imaging ($1 < \text{LogP/LogD} < 3$). As with DPA-714, cLogP value was much higher than LogP and LogD measurements.

In vitro stability in rat plasma

Stability of DPA-C5yne (**1**) was checked *in vitro* in rat spiked plasma at 37°C by LC–MS. Analyses of acetonitrile plasma extracts at different incubation time points (0, 5, 15, 60 and 90 min) showed only one single peak with a retention time (t_R: 12.9 min) and UV-spectrum ($\lambda_{max} = 270$ nm) identical to those of authentic DPA-C5yne. The variability in the peak area measurement as a function of time was less than 2%. Furthermore, for each time point, MS exhibited one molecular ion, at *m/z* 421 [$M + H^+$], with a mass spectrum and a fragmentation profile also identical to those of authentic DPA-C5yne. Thus, DPA-C5yne (**1**) proved to be stable in plasma at 37°C for at least 90 min because no decomposition product could be observed.

Autoradiography studies

Autoradiography studies were performed on slices of our in-house-developed rat model of acute local neuroinflammation. This model is based on an excitotoxic AMPA-mediated brain lesion in the right striatum of Wistar rats. It has already been validated for TSPO expression by immunohistochemical analyses[49–51] and used for the evaluation of several TSPO radioligands, such as [^{11}C]PK11195,[49,50] [^{11}C]CLINME,[50,52] [^{11}C]DPA-713,[49,51] [^{18}F]PBR111,[52] [^{18}F]DPA-714[51] and more recently [^{11}C]SSR180575.[53] Briefly, brain slices were incubated with [^{18}F]DPA-C5yne ([^{18}F]-**1**, 7.4 nM) alone, and also coincubated with non-labelled DPA-C5yne (**1**, 20 μM), or PK11195 (20 μM, the TSPO ligand of reference) or Flumazenil (20 μM, a CBR ligand of reference), respectively, in order to confirm the selectivity and specificity of the binding. As shown in Figure 3(A), a significantly increased binding of [^{18}F]DPA-C5yne ([^{18}F]-**1**) was detected in the lesion when compared to the control side. The target-to-background ratio (TBR), calculated as the bound tracer in the lesion versus the bound tracer in the contralateral side, was relatively high, 1.9 ± 0.3 ($p < 0.0001$). Addition of an excess of unlabelled DPA-C5yne or PK11195 (20 μM, 2700-fold mass excess compared to [^{18}F]DPA-C5yne) fully inhibited the binding (Figure 3(B) and 3(C)) in the lesioned area (TBR = 1.0 ± 0.1 for both ligands), proving a high specificity of the binding and selectivity for the TSPO. However, the addition of a similar excess of Flumazenil did not affect the binding of [^{18}F]DPA-C5yne (Figure 3(D)), yielding a calculated TBR of 1.9 ± 0.1, a value identical to the one found for [^{18}F]DPA-C5yne alone, proving its selectivity for the TSPO and not for the CBR.

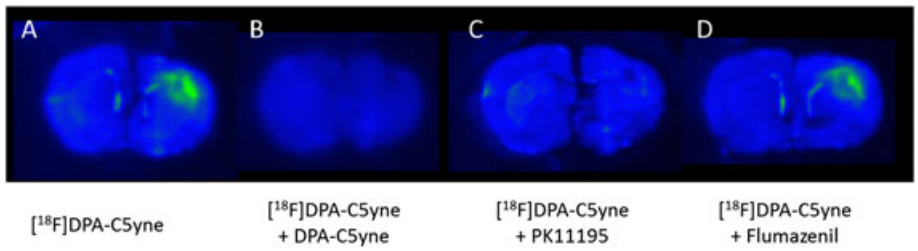


Figure 3. Autoradiography studies on slices of AMPA-lesioned rat brains incubated with [^{18}F]DPA-C5yne ([^{18}F]-**1**, 7.4 nM) alone (A) or with non-labelled DPA-C5yne (20 μM) (B), with PK11195 (20 μM) (C) and with Flumazenil (20 μM) (D), respectively.

Conclusions

The pyrazolo[1,5-*a*]pyrimidine-acetamide [^{18}F]DPA-C5yne is a novel, fluorine-18-labelled, radioligand targeting the TSPO with promising *in vitro* properties. PET imaging studies are currently being carried out in both acute and chronic animal models of neurodegeneration in order to evaluate the *in vivo* potential of this DPA-714 analogue.

Experimental

General

Chemicals

Chemicals were purchased from Aldrich, Fluka, Sigma or Cooper (France) and were used without further purification, unless otherwise stated.

Thin-layer chromatography analysis and flash chromatography

Thin-layer chromatography was run on pre-coated plates of silica gel 60F_{254} (VWR, Fontenay-sous-Bois, France). The compounds were localized when possible at 254 nm using a UV-lamp and/or by dipping the TLC-plates in a 1% ethanolic ninhydrin solution, a basic $KMnO_4$ aqueous solution or a 1% MeOH/H_2O (1/1, v:v) $FeCl_3$ solution and heating on a hot plate. Flash chromatographies were conducted on silica gel or alumina gel (0.63–0.200 mm, VWR) columns.

High-performance liquid chromatography analysis

[HPLC A]: Equipment: Waters (Guyancourt, France) system equipped with a 600E System Controller, a Prep LC 3000 pump, a 490E programmable multiwavelength UV-detector and a Kipp and Zonen (Emerainville, France) BD12E flatbed recorder; column: preparative Zorbax® RX-SIL, Hewlett Packard (Les Ulis, France) (250 × 21.2 mm); porosity: 5 μm; conditions: eluent: CH_2Cl_2/ MeOH: 98.5/1.5 [v:v]; flow rate: 10 mL/min; temperature: room temperature (RT); UV detection at λ: 230 nm. [HPLC B]: Equipment: Waters system equipped with a 510 pump and a Shimadzu SPD-10A UV-multiwavelength detector. Column: semipreparative SymmetryPrep® C-18, Waters (300 × 7.8 mm); porosity: 7 μm; conditions: eluent: CH_3CN/H_2O/TFA: 50/50/0.1 (v:v:v); flow rate: 5 mL/min; temperature: RT; UV detection at λ: 254 nm. [HPLC C]: Equipment: Waters system equipped with a 510 pump and a Shimadzu SPD-10A UV-multiwavelength detector. Column: semipreparative SymmetryPrep® C-18, Waters (300 × 7.8 mm); porosity: 7 μm; conditions: eluent: CH_3CN/H_2O/TFA: 60/40/0.1 (v:v:v); flow rate: 5 mL/min; temperature: RT; UV detection at λ: 254 nm. [HPLC D]: Equipment: TRACERLab FX N Pro (GE Medical Systems, Germany) integrated system, equipped with a Sykam S1122 Solvent Delivery System (pump), a Knauer K-2501 UV-multiwavelength detector and a miniaturized gamma-radioactivity detector. Column: semipreparative SymmetryPrep® C-18, Waters (300 × 7.8 mm); porosity: 7 μm; conditions: eluent: CH_3CN/H_2O/TFA: 60/40/0.1 (v:v:v); flow rate: 5 mL/min; temperature: RT; UV detection at λ: 254 nm. [HPLC E]: Equipment: Waters Alliance 2690 (or a Waters binary HPLC Pump 1525) equipped with a Waters 996 UV photodiode array detector and a Berthold LB509 radioactivity detector; column: analytical Symmetry-M® C-18, Waters (50 × 4.6 mm); porosity: 3.5 μm; conditions: eluent: solvA/solvB: 20/80 (v:v) [solvA: H_2O containing Low-UV PIC® B7 reagent (Waters), 20 mL for 1000 mL; solvB: H_2O/CH_3CN: 30/70 (v:v) containing Low-UV PIC® B7 reagent (Waters), 20 mL for 1000 mL]; flow rate: 2.0 mL/min; temperature: 30°C (or RT); UV detection at λ: 280 nm.

Spectroscopies

Nuclear magnetic resonance spectra (^{1}H/^{13}C) were recorded on a Bruker (Wissembourg, France) Avance (400 MHz) apparatus using the hydrogenated residue of the deuterated solvent $CDCl_3$ (δ = 7.23 ppm) as internal standard for ^{1}H-NMR as well as the deuterated solvent $CDCl_3$ (δ = 77.0 ppm) as internal standard for ^{13}C-NMR. The chemical shifts are reported in parts per million, downfield from Tetramethylsilane (TMS) (s, d, t, q, m, b for singlet, doublet, triplet, quadruplet, multiplet and broad, respectively). The MS were measured on a Thermo Scientific (Les Ulis, France) Ion Trap LCQ Deca XP + spectrometer (ESI+). The high resolution MS (HRMS) analyses were performed by Imagif (ICSN-CNRS, Gif-sur-Yvette, France) by electrospray with positive (ESI+) ionization mode on a Waters LCT Premier XE (Milford, MA, USA) spectrometer.

Radioisotope production

No-carrier-added fluorine-18 (half-life: 109.8 min) was produced *via* the [^{18}O(p,n)^{18}F] nuclear reaction by irradiation of a 2 mL [^{18}O]water (>97%-enriched, Rotem (CortecNet, Paris, France)) target on an IBA Cyclone-18/9 (IBA, Louvain-la-Neuve, Belgium) cyclotron (18 MeV proton beam), and the aqueous radioactive solution was then transferred to the appropriate hot cell. *Target hardware*: commercial, 2-mL, two-port, stainless steel target holder equipped with a domed-end niobium cylinder insert. *Target to hot cell liquid-transfer system*: 50 m Polytetrafluoroethylene (PTFE) line (0.8 mm internal diameter; 1/16 inch external diameter), 2.0 bar helium drive pressure and transfer time 2–3 min. Typical production of [^{18}F]fluoride at the end of bombardment for a 25 μA, 30 min (12.5 μAh) irradiation: 31.5–32.5 GBq.

Acute neuroinflammatory animal model

Striatal AMPA-mediated excitotoxicity and acute local neuroinflammation was induced in the brain of Wistar rats according to reported procedures.[49–51] The protocol used includes a stereotactic injection in the right striatum of 0.5 μL of AMPA ((*R,S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolopropionique, 15 mM in phosphate buffered saline buffer) in anaesthesized and normothermically controlled animals followed by a resting period of 7 days.

Chemistry

N,N-diethyl-2-(2-(4-(4-hydroxypent-1-yn-1-yl)phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)acetamide (3)

Synthesized in five steps from commercially available methyl 4-iodobenzoate according to reference.[46] Rf (CH_2Cl_2/MeOH: 95/5 v:v): 0.21. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.74 (d, 2H, *J* = 8.0 Hz, Ph), 7.46 (d, 2H, *J* = 8.0 Hz, Ph), 6.54 (s, 1H), 3.93 (s, 2H, CH_2), 3.81 (t, 2H, *J* = 6.4 Hz, $C\underline{H}_2OH$), 3.49 (q, 2H, *J* = 7.2 Hz, $NC\underline{H}_2CH_3$), 3.40 (q, 2H, *J* = 7.2 Hz, $NC\underline{H}_2CH_3$), 2.74 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.55 (t, 2H, *J* = 6.4 Hz, CH_2), 1.86 (q^5, 2H, *J* = 6.4 Hz, CH_2), 1.22 (t, 3H, *J* = 7.2 Hz, $NCH_2C\underline{H}_3$), 1.10 (t, 3H, J = 7.2 Hz, $NCH_2C\underline{H}_3$). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ 169.8 [C], 157.6 [C], 154.5 [C], 147.5 [C], 145.0 [C], 133.0 [C], 131.6 [2xCH], 128.4 [2xCH], 123.6 [C], 108.4 [CH], 101.3 [C], 90.2 [C], 81.1 [C], 61.7 [CH_2], 42.2 [CH_2], 40.6 [CH_2], 31.3 [CH_2], 28.0 [CH_2], 24.5 [CH_3], 16.8 [CH_3], 16.0 [CH_2], 14.3 [CH_3], 13.0 [CH_3]. MS (ESI+): *m/z* 419 $(M + H)^+$.

N,N-diethyl-2-(2-(4-(3-fluoropent-1-yn-1-yl)phenyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-3-yl)acetamide (1, DPA-C5yne)

To a solution of compound **3** (84 mg, 0.201 mmol) in dry CH_2Cl_2 (4 mL) was added a 50% Deoxofluor® solution in toluene (0.5 mL, excess). The reaction mixture was stirred for 3 days at RT, then diluted with MeOH (0.5 mL) and concentrated to dryness. Finally, the residue was purified by flash chromatography on silica gel (heptane/acetone: 4/1 (v:v)) to afford **1** (20 mg, 24%) as a yellow solid. For analytical purposes only, compound **1** was further purified using preparative HPLC [HPLC A] and obtained, after solvents removal, as a light yellow solid. Rf (heptane/acetone: 1/1 (v:v)): 0.35. t_R: 30 min [HPLC A], 10.5 min [HPLC B], 9.5 min [HPLC C], 9.5 min [HPLC D] and 2.20 min [HPLC E]. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.76 (d, 2H, *J* = 8.0 Hz, Ph), 7.47 (d, 2H, *J* = 8.0 Hz, Ph), 6.54 (s, 1H), 4.62 (dt, 2H, $J^2_{H\text{-}F}$ = 47.2 Hz, $J^3_{H\text{-}H}$ = 6.0 Hz, $C\underline{H}_2F$), 3.97 (s, 2H, CH_2), 3.50 (q, 2H, *J* = 7.2 Hz, $NC\underline{H}_2CH_3$), 3.41 (q, 2H, J = 7.2 Hz, $NC\underline{H}_2CH_3$), 2.76 (s, 3H, CH_3), 2.59 (t, 2H, *J* = 7.2 Hz, CH_2), 2.58 (s, 3H, CH_3), 2.01 (dq^5, 2H, $J^3_{H\text{-}F}$ = 25.6 Hz, $J^3_{H\text{-}H}$ = 6.0 Hz, CH_2), 1.23 (t, 3H, *J* = 7.2 Hz, $NCH_2C\underline{H}_3$), 1.12 (t, 3H, *J* = 7.2 Hz, $NCH_2C\underline{H}_3$). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ 169.7 [C], 157.6 [C], 154.5 [C], 147.4 [C], 145.2 [C], 133.0 [C], 131.6 [2xCH], 128.4 [2xCH], 123.5 [C], 108.4 [CH], 101.3 [C], 89.3 [C], 82.5 [d, $J^1_{C\text{-}F}$ = 164 Hz, CH_2], 81.2 [C], 42.2 [CH_2], 40.6 [CH_2], 29.5 [d, $J^2_{C\text{-}F}$ = 20 Hz,

CH_2], 28.0 [CH_2], 24.3 [CH_3], 16.9 [CH_3], 15.4 [d, $J^3_{C\text{-}F}$ = 4 Hz, CH_2], 14.3 [CH_3], 13.0 [CH_3]. MS (ESI+): *m/z*: 421 $(M+H)^+$. HRMS (*m/z*, ESI) calculated for $C_{25}H_{30}FN_4O$ (M + 1), 421.2404; found, 421.2404.

6-(4-(3-(2-(diethylamino)-2-oxoethyl)-5,7-dimethylpyrazolo[1,5-a]pyrimidin-2-yl)phenyl)pent-4-yn-1-yl)4-methylbenzenesulfonate (2)

To a solution of compound **3** (200 mg, 0.478 mmol) in dry CH_2Cl_2 (5 mL) was added Et_3N (80 µL, 0.578 mmol, 1.2 eq) and DMAP (6 mg, 0.049 mmol, 0.1 eq). The flask was degased and flushed with argon (two cycles), then cooled to 0°C. 4-toluenesulfonic anhydride (170 mg, 0.521 mmol, 1.1 eq.) was slowly added to the previous solution, and the reaction mixture stirred at 0°C for 15 min, then at RT overnight. 4-toluenesulfonic anhydride (50 mg, 0.153 mmol, 0.3 eq) and Et_3N (100 µL, 0.71 mmol, 1.5 eq) were added once again, and the mixture stirred another 2 days at RT. The reaction mixture was then evaporated under reduced pressure, and the resulting brownish oil was portioned between EtOAc (20 mL) and aq 0.05 M HCl (15 mL). The organic layer was separated, washed with water (10 mL), brine (10 mL), dried over Na_2SO_4, filtered and concentrated to dryness. Finally, the resulting residue was purified by flash chromatography on silica gel (pure CH_2Cl_2 to CH_2Cl_2/MeOH: 98/2 (v:v)) to afford **2** (145 mg, 54%) as a light yellow solid. Rf (CH_2Cl_2/MeOH: 93/7 v:v): 0.58. t_R: 21.5 min [HPLC B], 15.5 min [HPLC C], 15.3–15.9 min [HPLC D], 3.51 min [HPLC E]. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.80 (d, 2H, *J* = 8.0 Hz, Ph), 7.76 (d, 2H, *J* = 8.4 Hz, Ph), 7.36 (d, 2H, *J* = 8.4 Hz), 7.31 (d, 2H, *J* = 8.0 Hz, Ph), 6.53 (s, 1H), 4.21 (t, 2H, *J* = 5.6 Hz, $C\underline{H}_2OTs$), 3.94 (s, 2H, CH_2), 3.51 (q, 2H, *J* = 7.2 Hz, $NC\underline{H}_2CH_3$), 3.40 (q, 2H, *J* = 7.2 Hz, $NC\underline{H}_2CH_3$), 2.74 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 2.49 (t, 2H, *J* = 6.8 Hz, CH_2), 2.39 (s, 3H, CH_3), 1.94 (q^5, 2H, *J* = 6.8 Hz, CH_2), 1.23 (t, 3H, *J* = 7.2 Hz, $NCH_2C\underline{H}_3$), 1.11 (t, 3H, *J* = 7.2 Hz, $NCH_2C\underline{H}_3$). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ 169.7 [C], 157.6 [C], 154.4 [C], 147.0 [C], 145.0 [C], 144.8 [C], 133.1 [C], 132.8 [C], 131.6 [2xCH], 129.8 [2xCH], 128.4 [2xCH], 127.8 [2xCH], 123.3 [C], 108.4 [CH], 101.3 [C], 88.4 [C], 81.7 [C], 68.9 [CH_2], 42.2 [CH_2], 40.6 [CH_2], 28.0 [CH_2], 27.9 [CH_2], 24.3 [CH_3], 21.6 [CH_3], 16.8 [CH_3], 15.7 [CH_2], 14.3 [CH_3], 13.0 [CH_3]. MS (ESI+): *m/z*: 573 $(M+H)^+$. HRMS (*m/z*, ESI) calculated for $C_{32}H_{37}N_4O_4S$ (M + 1), 573.2536; found, 573.2524.

Radiochemistry

The TRACERLab FX N Pro synthesizer: description, configuration, preparation and sequences used

Synthesizer description and sequences used

Fluorine-18-labelling, purification with HPLC and formulation of [^{18}F]DPA-C5yne ([^{18}F]-**1**) as an *i.v.* injectable solution was performed using a commercially available TRACERLab FX N Pro synthesizer (GE Medical Systems, Germany), placed in a 5.0-cm-lead shielded and ventilated cell. The whole process was thus fully automated and used a dedicated method supported by three generic 'time list', written according to the programming mode of General Electric synthesis modules, and named, respectively, 'Time list 1: K[^{18}F]F-K_{222} preparation', 'Time list 2: radiofluorination and pre-purification' and 'Time list 3: purification and formulation'.[12] Before each [^{18}F]DPA-C5yne ([^{18}F]-**1**) batch production, the synthesizer was cleaned out (e.g. used-cartridge removal), purged from any residual chemicals or solvents left-over (reservoirs, reactors and tubings) from a previous radiotracer production and dried using another in-house programmed method, based on the use of two generic 'time list', named, respectively, 'Time list: clean' and 'Time list: clean and dry'.[12] The first cleaning procedure used deionized water to rinse both reactors [Reactor 1–2], the flask collecting the oxygen-18-enriched water [Water collect] and the tube dedicated to the HPLC injection [HPLC tube] and most of the connecting tubings. The second procedure used both acetone and ethanol to further clean the synthesizer but also to dry all reservoirs [Reservoir 1–14], both reactors [Reactor 1–2], the tube dedicated to the HPLC injection [HPLC tube], the flask allowing the dilution of the HPLC-collected fraction prior to cartridge formulation [Dilution flask] and the flask required for receiving at last the batch of formulated product [Product vial] as well as all tubings connecting these elements.

Synthesizer configuration

Valves VZ1 and VZ2 were both turned in up-position for the preparation of [^{18}F]DPA-C5yne ([^{18}F]-**1**), therefore by-passing most of the added functionalities of the TRACERLab FX N Pro synthesizer when compared to the TRACERLab FX FN one (e.g. the second reactor [Reactor 2] and its connected reservoirs [Reservoir 7–11] and associated tubings). Note that valves VX3 and VX4 as well as the associated option of using an additional cartridge purification unit were also not requested for this process.

Synthesizer preparation

Prior to [^{18}F]DPA-C5yne ([^{18}F]-**1**) production, the following reservoirs and vessels were filled with the solutions described here: [Reservoir 1]: a mixture of deionized water and acetonitrile (30/70 v/v, 1 mL) containing 1.5 mg of K_2CO_3 and 12–15 mg of Kryptofix®222; [Reservoir 3]: a DMSO solution (0.7 mL) containing the tosylate **2** (4.0–5.0 mg, 7.0–8.7 µmol), as the labelling precursor; [Reservoir 4]: HPLC solvent (2 mL, see [HPLC D]); [Reservoir 5]: HPLC solvent (2 mL, see [HPLC D]; [Reservoir 12]: deionized water (10 mL); [Reservoir 13]: ethanol (1.5 mL); [Reservoir 14]: aq 0.9% NaCl (8.5 mL); [Dilution flask]: deionized water (20 mL). Note that [Reservoir 2], [Reservoir 6], [Reservoir 7–11], [Reactor 1], [Reactor 2], [Product vial], [HPLC tube], [[^{18}O] Water collect], [Water waste] and all other [WASTE] were left empty. Additionally, all cartridges requested for the preparation of [^{18}F]DPA-C5yne ([^{18}F]-**1**) were, before being mounted on the synthesizer, conditioned as described here: [QMA cartridge]: Sep-Pak® Light Accell™ Plus QMA cartridge (Waters), hydroxide form, generated from the chloride form by washing with aq 1M $NaHCO_3$ (2 mL) and rinsed with deionized water (20 mL) and CH_3CN (10 mL); [Alumina N cartridge]: Sep-Pak® Alumina N™ cartridge (Waters), washed with deionized water (10 mL); [C-18 cartridge]: Sep-Pak®Plus C18 cartridge (Waters), washed with EtOH (2 mL) and then rinsed with deionized water (10 mL). Finally, the HPLC-column and system was also conditioned with the appropriate solvent (see [HPLC D]), and DPA-C5yne (**1**), as standard, was injected once.

Radiosynthesis, purification and formulation of [^{18}F]DPA-C5yne ([^{18}F]-1)

K[^{18}F]F-K_{222} preparation (Time list 1)

The target content (oxygen-18-enriched water containing [^{18}F] fluorine-18) was transferred to the TRACERLab FX N Pro synthesiser using helium pressure (2 bars) and was first collected in the [[^{18}O]-water collect] vial, then sucked through the [QMA cartridge]. At this stage, fluorine-18 was trapped, as [^{18}F]fluoride, in the cartridge, and the oxygen-18-enriched water was collected in the [Water waste] vial. [^{18}F]fluoride anions were then released from the cartridge using the solution stored in [Reservoir 1] and eluted into [Reactor 1]. The mixture was gently concentrated to dryness using the following two heating steps sequentially: (i) 60°C, 7 min at a pressure between 30 and

35 kPa, then (ii) 120°C, 5 min under vacuum. Additional dilution with CH_3CN followed by azeotropic distillation was not performed. The expected K[^{18}F]F-K_{222} complex, ready for the fluorination step, was obtained within 18 min.

Fluorine-18 incorporation and pre-purification (Time list 2)

The content of the [Reservoir 3] (e.g. 4–5 mg of **2** as precursor for labelling in solution in 700 μL of DMSO) was added to the [Reactor 1] (the latter containing the aforementioned K[^{18}F]F-K_{222} complex). The [Reactor 1] was then heated at 100°C for 10 min and then cooled down to 50°C. The content of [Reservoir 4] (HPLC solvent, 2 mL) was added to the [Reactor 1]. The diluted content of **[**Reactor 1] was then transferred under pressure through the [Alumina cartridge] and collected in the [HPLC tube]. The content of **[**Reservoir 5] (HPLC solvent, 2 mL) was further added to the **[**Reactor 1] and the novel content of **[**Reactor 1] was then analogously transferred (under pressure) through the **[**Alumina cartridge**]** and collected in the **[**HPLC tube**]** too. The part of the process described here lasted about 17 min.

High-performance liquid chromatography purification and formulation (Time list 3)

The cartridge-purified solution (about 4.7 mL) collected in the **[**HPLC tube**]** was transferred under pressure into the HPLC injection loop (5 mL). HPLC purification started at the signal given by the fluid detector, the latter switching the loop-valve from the 'load' position to the 'inject' position. HPLC elution was followed by both UV (254 nm) and radioactivity detection. The fraction containing [^{18}F]DPA-C5yne ([^{18}F]-**1**) was collected separately, on the operator's actions (clicking on the start-collect and end-collect icons), in the **[**Dilution flask**]**, prefilled with water (20 mL). Final formulation of the radiotracer was performed automatically using a Sep-Pak® Plus C18-based system. For this, the content of the **[**Dilution flask**]** was first passed through the [C-18 cartridge**]** (trapping the radiotracer), the latter being then washed with water (10 mL, stored in the **[**Reservoir 12]. Elution of the cartridge with EtOH (1.5 mL, stored in the **[**Reservoir 13]) afforded [^{18}F]DPA-C5yne ([^{18}F]-1), which was recovered in the **[**Product flask**]**. Addition of 0.9% aq NaCl (8.5 mL, stored in the **[**Reservoir 14]) through the [C-18 cartridge**]** was performed at last to complete the formulation step. HPLC purification and formulation steps lasted about 20–25 min.

Quality control of formulated [^{18}F]DPA-C5yne ([^{18}F]-1)

Appearance test

The radiotracer preparation was visually inspected behind a lead-shielded glass window for clarity and absence of particulates.

pH test

The pH was determined using standard pH paper using an aliquot of the radiotracer preparation.

Identification and chemical and radiochemical purity test

Identification as well as chemical and radiochemical purity determinations were assessed by separate analytical-HPLC analysis, using a sample of authentic **1** as standard (particular attention was paid to the absence of non-radioactive precursor **2** (tosyloxy derivative)). For this, an aliquot of the radiotracer preparation (5–20 μL) was injected onto HPLC [HPLC E]. The chemical identity was determined by UV-spectrometric analysis, by comparison of the UV-profile, λ_{max} and retention time recorded with those observed for the corresponding reference compound (**1**) in the same HPLC system and conditions. Chemical purity was also assessed on the UV-chromatogram based on the relative integration of the peak areas. Radiochemical identity was also confirmed by combined radioactive and UV-spectrometric analysis, by comparison of the retention time of the radioactive compound with the corresponding non-radioactive reference compound (**1**). Radiochemical purity was assessed on the radioactive chromatogram based on the relative integration of the peak area corresponding to the radiotracer.

Specific radioactivity determination

Specific radioactivity of the radiotracer was calculated from three consecutive HPLC analyses. For this, an aliquot of the radiotracer preparation (5–20 μL) was injected onto HPLC [HPLC E], and for each injection, the area of the UV absorbance peak corresponding to the product was measured (integrated) on the HPLC chromatogram and compared to a standard curve relating mass to UV absorbance, allowing the determination of the corresponding mass. The HPLC fraction corresponding to the peak was also collected in a vial, and its radioactivity measured in an ionization chamber (Capintec, Ramsey, NJ, USA). The specific radioactivity, as a mean of three independent experiments, was calculated by dividing the counted radioactivity by the found mass (triplicate).

On-shelf stability test

Chemical and radiochemical stability of the entire preparation was checked by HPLC. For this, aliquots of the radiotracer preparation (20 μL) were injected onto HPLC [HPLC E] at regular 15-min intervals during 120 min. For each injection, the chemical and radiochemical purity was determined (see in the preceding text). Moreover, for each injection, the mass associated to the peak corresponding to the product was calculated (using the standard curve relating mass to UV absorbance), and its radioactivity was also measured, before being then corrected for the decay of fluorine-18. Values of both mass and decay-corrected radioactivity per sample were plotted against time and fitted for linearity.

Lipophilicity determination

LogP (*n*-octanol/water partition coefficient)

[^{18}F]DPA-C5yne ([^{18}F]-**1,** 1–5 kBq in 50 μL of water) was added to a two-layer system of *n*-octanol (500 μL) and water (450 μL) in an Eppendorf cap (VWR, Fontenay-sous-Bois, France). The vessel was strongly vortexed for 3 min and then centrifuged at 3000 rpm for 2 min. An aliquot of each layer (100 μL) was assessed for radioactivity in a cross-calibrated Perkin-Elmer Cobra Quantum γ-counter (Les Ulis, France).

LogD (*n*-octanol/buffer pH 7.4 partition coefficient)

The procedure described earlier was repeated by replacing water by 0.1 M phosphate buffered saline pH 7.4 (450 μL). The partition coefficients (LogP and LogD) were calculated as the decimal logarithm of the ratio between the counted radioactivity in the *n*-octanol phase and the counted radioactivity in the aqueous phase.

CLogP (calculated LogP)

CLogP values were determined using ChemBioDraw Ultra 11.0.1 software (Cambridge Soft., Perkin-Elmer, Waltham, MA, USA).

In vitro stability in rat plasma

Sample preparation

Rat plasma (4 mL, prepared from whole blood by centrifugation (5 min, 3000 g and at 4°C)) was spiked with 40 μL of a 10 mM DPA-C5yne (**1**) solution in CH_3CN (final concentration: 100 μM, maximum 1% of organic solvent). Two samples of 200 μL were first removed, and an equal volume of CH_3CN (200 μL) was added. For each sample, the mixture was centrifuged (4°C, 3500 rpm, 5 min) and the supernatant was collected. 20 μL of each extract were then injected onto the LC–MS system, and the peak area corresponding to DPA-C5yne was measured (time zero). The remainder of the spiked plasma was then incubated at 37°C for 5, 15, 30, 60 and 90 min. For each time point, two samples were removed and processed as indicated earlier.

LC–MS analyses

Liquid chromatography–mass spectrometry analyses were performed using an Ion Trap LCQ Deca XP + mass spectrophotometer equipped with an electrospray source (Thermo Scientific, Les Ulis, France). Pressurized nitrogen was used as sheath gas with a flow rate of 25 units (arbitrary units for sheath gas pressure as defined by the manufacturer). The source voltage for ESI was 4.5 kV and the capillary voltage was 38 V. The capillary temperature was 275°C. The HPLC system (Thermo Scientific) interfaced with the mass spectrophotometer consisted of a Surveyor pump (54949 series), a Surveyor autosampler (55989 series), a photodiode array detector (56470 series) and an analytical Atlantis C18 (Waters) column (2.1 × 150 mm, porosity 5 μm). The mobile phase consisted of A: H_2O containing 0.05% formic acid and B: CH_3CN containing 0.05% formic acid. A linear gradient from 40 to 80% of B in 10 min was applied to the column at a flow rate of 200 μL/min and returns to the initial conditions within 5 min. The whole output of the LC column was passed through the photodiode array detector (190–600 nm) before ESI probe of the mass spectrometer (operated in the positive mode). In the full scan MS acquisition mode, the instrument method was set up to detect ions in the range of *m/z* 50–500. Data acquisition and processing were performed with Xcalibur™ software (version 2.0, Thermo Scientific, Les Ulis, France). The peak area corresponding to DPA-C5yne (**1**) was measured as a function of the incubation time.

Brain autoradiography studies with [^{18}F]DPA-C5yne ([^{18}F]-1)

Brain slices of unilaterally AMPA-lesioned animals were prepared according to reported procedures.[49–51] The protocol used includes the decapitation of the animals under terminal anaesthesia, quick brain removal and freezing in cold (−80°C, dry-ice) isopentane followed by coronally, 10 μm thick slicing of the brain at the level of the lesion. Then, adjacent brain slices (36 slices from the centre of the lesioned area) were taken and incubated for 20 min in Tris Buffer (TRIZMA pre-set Crystals, Sigma®, adjusted at pH 7.4 at 4°C, 50 mM with NaCl 120 mM) containing [^{18}F]DPA-C5yne (111 MBq, 75 GBq/μmol, 555 MBq/L, 7.4 nM) alone, or [^{18}F]DPA-C5yne and PK11195 (20 μM), [^{18}F]DPA-C5yne and DPA-C5yne (20 μM) as well as [^{18}F]DPA-C5yne and Flumazenil (20 μM). Brain sections were then washed two times for 2 min and once for 10 s with cold (4°C) buffer, then exposed on a Phosphor-Imager (Storm 860, Molecular Dynamics, Pharmacia, GE Medical systems, Germany) screen overnight. Autoradiograms were scanned and then analysed using the ImageJ software (developed by the National Institutes of Health). A region of interest was manually drawn around the core of the lesion, and an identical area was copy-pasted symmetrically into the contralateral hemisphere. Binding in the region of interest was then expressed as the number of counts per surface unit. The TBR was calculated as the ratio of the binding in the lesioned versus the contralateral hemisphere.

Acknowledgements

The authors wish to thank the cyclotron operators Mr Daniel Gouel, Mr Christophe Lechêne and Mr Tony Catarina for performing the irradiations. This work was supported by the CEA-I^{2}BM intramural programmes, as well as the European Union's Seventh Framework Programme [FP7/2007–2013] INMiND (Grant agreement no HEALTH-F2-2011-278850). PhD students Vincent Médran-Navarrete and Nicholas Bernards were in part supported by a CEA Irtelis doctoral grant and the EU FP7 INMiND programme (see in the preceding text), respectively.

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