

Fully automated radiosynthesis of N^1 -[^{18}F]fluoroethyl-tryptophan and study of its biological activity as a new potential substrate for indoleamine 2,3-dioxygenase PET imaging

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ABSTRACT

Introduction:

Indoleamine 2,3-dioxygenase (IDO) catalyzes the initial step in the catabolism of L-tryptophan along the kynurenine pathway and exerts immunosuppressive properties in inflammatory and tumor tissues by blocking locally T-lymphocyte proliferation. Recently, 1-(2-[^{19}F]fluoroethyl)-DL-tryptophan (1-[^{19}F]FE-DL-Trp) was reported as a good and specific substrate of this enzyme. Herein, the radiosynthesis of its radioactive isotopomer (1-[^{18}F]FE-DL-Trp, DL-[^{18}F]5) is presented along with *in vitro* enzymatic and cellular uptake studies.

Methods:

The one-pot n.c.a. radiosynthesis of this novel potential PET imaging tracer, including HPLC purification and formulation, has been fully automated on a FASTLabTM synthesizer. Chiral separation of both isomers and their formulation were implemented on a second cassette. *In vitro* enzymatic and cellular uptake studies were then conducted with the D-, L- and DL-radiotracers.

Results:

The radiolabeling of the tosylate precursor was performed in DMF (in 5 min; RCY: 57% (d.c.), n=3). After hydrolysis, HPLC purification and formulation, DL-[^{18}F]5 was obtained with a global radiochemical yield of 18±3% (not decay corrected, n=7, in 80 min) and a specific activity of 600±180 GBq/μmol (n=5). The subsequent separation of L- and D-enantiomers was performed by chiral HPLC and both were obtained after formulation with a RCY (d.c.) of 6.1% and 5.8%, respectively. *In vitro* enzymatic assays reveal that L-[^{18}F]5 is a better substrate than D-[^{18}F]5 for human IDO. *In vitro* cellular assays show an IDO-specific uptake of the racemate varying from 30% to 50% of that of L-[^{18}F]5, and a negligible uptake of D-[^{18}F]5.

Conclusion:

In vitro studies show that L-[^{18}F]5 is a good and specific substrate of hIDO, while presenting a very low efflux. These results confirm that L-[^{18}F]5 could be a very useful PET radiotracer for IDO expressing cells in cancer imaging.

Keywords:

Tryptophan
PET imaging
Indoleamine 2,3-dioxygenase
1-(2-[^{18}F]fluoroethyl)tryptophan
Fully automated radiosynthesis
FASTLab

1. Introduction

Human indoleamine 2,3-dioxygenase (hIDO) is, with tryptophan 2,3-dioxygenase (TDO), one of the two main enzymes that catalyze, through the kynurenine pathway, the first and rate-limiting step of the irreversible oxidative ring opening of the indole nucleus of L-tryptophan, one of the least abundant of the essential amino acids.[1–4] This heme-containing enzyme is mainly expressed in placenta (preventing the rejection of allogeneic fetuses), in inflammatory tissues, and in human tumor

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cells (such as endometrial, cervical, kidney, non-small cell lung, stomach, mammary, prostatic, and pancreatic carcinomas).[3,5] As a consequence, tryptophan depletion and accumulation of its catabolites are observed in these tissues. Both metabolic modifications result in a local immunosuppressive microenvironment following several potential mechanisms (such as T-cell proliferation blocked by L-tryptophan shortage and T-cell apoptosis due to accumulated L-tryptophan catabolites) whose respective role still remains incompletely characterized.[1,6–8]

Recently, our group has demonstrated that 1-(2-[¹⁹F]fluoroethyl)-DL-tryptophan (1-[¹⁹F]FE-DL-Trp) is a good and specific substrate of the recombinant human indoleamine 2,3-dioxygenase (rhIDO). We have also suggested that its radioactive isotopomer, 1-[¹⁸F]FE-DL-Trp (DL-[¹⁸F]**5**), with its relatively long half-life time ($[^{18}\text{F}]t_{1/2} = 109.7$ min), could be a highly desirable and valuable tool to facilitate i) the preclinical and clinical development and validation of new hIDO inhibitors, and ii) the clinical detection of hIDO expressing cells in cancer imaging.[5] According to these results, the radiosynthesis of 1-[¹⁸F]FE-DL-Trp was considered.

A multistep radiosynthesis of this radiotracer proceeding with very limited radiochemical yield (RCY about 1%) was previously described by Sun and co-workers.[9] Therefore, we report here our efforts to develop a more appropriated synthesis for future animal or human PET investigations. Our strategy implies a simplified two-steps no-carrier-added (n.c.a.) radiosynthesis of 1-[¹⁸F]FE-DL-Trp (DL-[¹⁸F]**5**) by means of a fast and regioselective labeling of a fully protected tosylate precursor. The whole process was fully automated on a *GE Healthcare* FASTlabTM system utilizing single use cassettes.[10] Furthermore, a chiral HPLC separation of the two enantiomers and their automated formulation was also implemented.

Finally, the results of some *in vitro* enzymatic and cellular assays (including affinity, specificity, uptake and efflux) with 1-[¹⁸F]FE-DL-Trp (DL-[¹⁸F]**5**) and its two resolved enantiomers, 1-[¹⁸F]FE-L-Trp (L-[¹⁸F]**5**) and 1-[¹⁸F]FE-D-Trp (D-[¹⁸F]**5**), were compared.

2. Material and methods

2.1. Chemistry – Generality

All solvents (HPLC grade), chemicals and reagents (analytical grade) were purchased from Sigma Aldrich, Merck or VWR, and were used without further purification. HPLC analyses were run on a Waters system (600 pump, 996 PDA UV detector (200 – 400 nm)) controlled by the Empower software. Analytical HPLC analyses were performed on X-Terra[®] RP18 column (150 x 4.6 mm, 3.5 μm ; Waters), with MeCN and H₂O containing 0.1% TFA at 0.7 mL.min⁻¹. Semi-preparative HPLC analyses were carried out on X-Terra[®] Prep RP18 column (10 x 250 mm, 10 μm ; Waters) at 4 or 5 mL.min⁻¹. Chiral HPLC analyses were done on Astec ChirobioticTM T column (25 cm x 4.6 mm, 5 μm ; MeOH/H₂O 85/15 (v/v), 0.7 mL.min⁻¹). UPLC analyses were run on a Waters system (Acquity UPLC[®] PDA UV e λ detector (190 – 400 nm) controlled by the Empower software) and were performed on ACQUITY UPLC[®] BEH C18 column (2.1 x 100mm, 1.7 μm ; Waters), at 0.7 mL.min⁻¹ and 50°C. Thin layer chromatography (TLC) analyses were carried out on silica gel Polygram[®] SIL G/UV₂₅₄ pre-coated TLC-sheets. ¹H, ¹³C and ¹⁹F NMR spectra were recorded at room temperature on a Bruker 250 MHz or 400 MHz. Chemical shifts δ were given in ppm, and the multiplicity patterns were abbreviated as: s = singlet, d = doublet, t = triplet, q = quadruplet, p = quintet, m = multiplet, br = broad. The coupling constant *J* was given in Hz. Spectra were recorded as solutions in d₁-CDCl₃ (d_H at 7.26 ppm, d_C at 77.16 ppm), d₄-MeOD (d_H at 3.31 ppm, d_C at 49.00 ppm), or d₂-D₂O (d_H at 4.79 ppm) which were used as internal references. The melting point of solid compounds was determined with a Melting Point (Mp) Instrument Digital 9000 Series IA9100 230V. Mass spectra (MS) were recorded on a Finnigan Thermoquest TSQ7000 mass spectrometer (ThermoElectronCorp.) operating in full-scan MS mode with an electrospray source (ESI+/-). FT-MS mass analyses were performed on an ESI-FT-ICR mass spectrometer (Solarix, Bruker) in positive ion mode. Extern calibration was done over the range of *m/z* 50 to 1500 using H₃PO₄ adducts and mean residual error obtained was < 0,5 ppm. Internal correction was applied

using GluFib peptide standard (Waters). Samples were analyzed at 13 μ M in H₂O/ACN (50/50), formic acid 0.1% solution supplemented with 0.33 μ M GluFib.

The syntheses of *tert*-butoxycarbonyl-*L*-tryptophan *tert*-butyl ester (**2**), 1-fluoro-2-tosyloxyethane (**3**), *tert*-butoxycarbonyl-(1-(2-fluoroethyl)tryptophan) *tert*-butyl ester ([¹⁹F]**4**) and 1-(2-fluoroethyl)-DL-tryptophan chlorhydrate (1-FETrp.HCl) (DL-[¹⁹F]**5**) were done according to literature procedures.[5]

2.1.1. Synthesis of *tert*-butoxycarbonyl-(1-(2-tosyloxyethyl)-DL-tryptophan) *tert*-butyl ester (**7**)

tert-Butoxycarbonyl-*L*-tryptophan *tert*-butyl ester **2** (371 mg, 1.03 mmol) in DMF (3 mL) was added dropwise and over 12 minutes to a solution of sodium hydride (240 mg, 6 mmol) and 1,2-ditosyloxyethane **6** (3.708 g, 10 mmol) in DMF (32 mL), cooled beforehand at 0°C for 20 minutes. The mixture was vigorously shaken for 15 minutes at 0°C, after which NH₄Cl aqueous solution (5%wt) was slowly added. The crude product was extracted with EtOAc (3 x 30 mL). The organic layers were dried over anhydrous MgSO₄, and after filtration, the solvent was removed under reduce pressure. Acetone was added to the residue and a part of the excess of **6** was filtered off. The filtrate was concentrated and finally purified by semi-preparative HPLC (MeCN/H₂O 60/40 (v/v)) to give *tert*-butoxycarbonyl-(1-(2-tosyloxyethyl)-DL-tryptophan) *tert*-butyl ester **7** as colorless oil (282 mg, 49%). R_f (Hexanes/EtOAc 2/1 (v/v)): 0.33; R_f (CH₂Cl₂/EtOAc 9/1 (v/v)): 0.73. ¹H NMR (250 MHz, CDCl₃) δ 7.60 – 7.38 (m, 3H, CH_{arom}), 7.16 – 7.00 (m, 5H, CH_{arom}), 6.83 (s, 1H, CH_{indol}), 5.07 (d, *J* = 7.9 Hz, 1H, NH_a), 4.49 (dd, *J* = 12.8, 5.5 Hz, 1H, CH_a), 4.31 – 4.20 (m, 4H, CH₂CH₂-OTs), 3.17 (qd, *J* = 14.8, 5.6 Hz, 2H, CH₂), 2.32 (s, 3H, CH₃), 1.41 (d, *J* = 8.5 Hz, 18H, CH₃ Boc and tBu). ¹³C NMR (63 MHz, CDCl₃) δ 171.26 (CO), 155.26 (CO), 144.80 (C_{TsO}), 135.83 (C_{TsO}), 131.91 (C_{arom}), 129.64 (2 CH_{TsO}), 128.73 (C_{arom}), 127.54 (2 CH_{TsO}), 126.56 (CH_{indol}), 121.96 (CH_{arom}), 119.36 (CH_{arom}), 119.34 (CH_{arom}), 110.18 (C_{indol}), 108.83 (CH_{arom}), 81.83 – 79.55 (C_{tBu}), 67.85 (CH₂-OTs), 54.72 (CH_a), 44.97 (CH₂CH₂-OTs), 28.33 (CH₂), 27.96 (CH₃ Boc and tBu), 21.57 (CH₃). MS: (ESI, positive): *m/z* = 559 [M+H] and 581 [M+Na]. FT-MS (positive): *m/z* = 559.2472 (calculated), 559.2464 (measured) [M+H].

2.1.2. Synthesis of 1-(2-fluoroethyl)-DL-tryptophan methyl ester (DL-[¹⁹F]**8**)

A solution of 1-(2-fluoroethyl)-DL-tryptophan (DL-[¹⁹F]**5**) (86 mg, 0.3 mmol) in MeOH (10 mL) and TMSCl (2 mL) was stirred at room temperature for 24h. After evaporation of the large excess of reactants under reduce pressure, the crude product was crystallized in Et₂O/MeOH to provide DL-[¹⁹F]**8** as an off-white solid (74 mg, 82%). ¹H NMR (400 MHz, MeOD) δ 7.58 (d, *J* = 7.9 Hz, 1H, CH_{arom}), 7.46 (d, *J* = 8.3 Hz, 1H, CH_{arom}), 7.23 (t, *J* = 7.7 Hz, 1H, CH_{arom}), 7.22 (s, 1H, CH_{indol}), 7.13 (t, *J* = 7.5 Hz, 1H, CH_{arom}), 4.73 (dt, ²*J*_{H-F} = 47.4 Hz and ³*J*_{H-H} = 4.7 Hz, 2H, CH₂-F), 4.48 (dt, ³*J*_{H-F} = 27.3 Hz and ³*J*_{H-H} = 4.7 Hz, 2H, CH₂CH₂-F), 4.35 (t, *J* = 6.5 Hz, 1H, CH_a), 3.80 (s, 3H, CH₃), 3.46 – 3.34 (m, 2H, CH₂, β). ¹³C NMR (101 MHz, MeOD) δ 170.76 (CO), 138.31 (CH_{arom}), 129.31 (C_{arom}), 128.91 (CH_{arom}), 123.26 (CH_{arom}), 120.72 (CH_{arom}), 119.27 (CH_{arom}), 110.93 (CH_{arom}), 107.65 (C_{arom}), 83.91 (d, ²*J*_{C-F} = 169.51 Hz, CH₂F), 53.64 (CH_a), 47.53 (d, ³*J*_{C-F} = 20.69 Hz, CH₂CH₂F), 27.41 (CH₂, β). ¹⁹F NMR (376 MHz, MeOD) δ -221.61 (tt, ²*J*_{H-F} = 47.5 Hz and ³*J*_{H-F} = 27.3 Hz). MS: (ESI, positive): *m/z* = 265 [M+H], 287 [M+Na]. Mp: 197-199°C.

2.1.3. Synthesis of 1-(2-fluoroethyl)-L-tryptophan (L-[¹⁹F]**5**)

α -Chymotrypsin (45 μ L) from bovine pancreas Type II (2.5 mg/mL (ammonium formate buffer, 100 mM); lyophilized powder; \geq 40 units/mg protein) was added to DL-[¹⁹F]**8** (8.5 mg, 27.5 mM) in ammonium formate buffer (1.1 mL, 100 mM, pH 5.4). The reaction, carried out at 37°C, was followed in HPLC (RP18; H₂O + 0.05% TFA/ACN/H₂O (1/10/89) (v/v/v); 0.7 mL.min⁻¹). After 5 hours, the reaction was completed and the pH of the solution had decreased to 4.6. An aliquot (100 μ L) was diluted with buffer (200 μ L) and filtered with VWR centrifugal filter-modified PES 3k (500 μ L). The filtrate was then purified by HPLC (RP18). The peak of the hydrolyzed compound L-[¹⁹F]**5** was collected and analyzed on a chiral column (Astec CHIROBIOTICTM T (MeOH/H₂O (85/15) (v/v); 0.7 mL.min⁻¹).

2.2. Radiosynthesis – Generality

Semi-preparative HPLC analyses were performed on X-Terra® Prep RP18 column (10 x 250mm, 10 µm, Waters), with EtOH/sodium acetate buffer (100 mM, pH 4) 5/95 (v/v) at 5 mL.min⁻¹ (*condition A*). Chiral HPLC analyses were carried out on an Astec Chirobiotic™ T column (25 cm x 4.6 mm, 5 µm) with a mixture of MeOH/H₂O (85/15 (v/v)) at a flow rate of 0.7 mL.min⁻¹ (*condition B*). UPLC analyses were run at 50°C on ACQUITY UPLC® BEH C18 column (2.1 x 100mm, 1.7µm; Waters) on an Acquity UPLC® system (Waters), using either a MeCN/H₂O + 0.1% TFA gradient (from 80/20 to 20/80 (v/v) in 4 min, at 0.7 mL.min⁻¹ (*condition C*)) or a MeCN/H₂O + 0.1% TFA isocratic mixture (20/80 (v/v), at 0.7 mL.min⁻¹ (*condition D*)) as eluent. The UV signal of the products were measured at 221 nm and 284 nm with a PDA UV detector (190 – 400 nm) controlled by the Empower software and connected to the HPLC or UPLC system. The radioactive elution profiles were monitored with a custom-made Geiger-Müller (GM) radioactivity detector connected after PDA UV detector to the HPLC or UPLC system. TLC analyses were done on silica gel Polygram® SIL G/UV₂₅₄ pre-coated TLC-sheets (TLC eluent conditions: n-hexane/EtOAc 1/1 (v/v) (*condition E*), and ACN/H₂O/MeOH 4/1/1 (v/v/v) (*condition F*). A Bioscan TLC scanner model AR2000 was used for analysis of the ¹⁸F labeled compounds. The FASTlab™ is a commercially available synthesizer using single used cassette. This module was developed by GE Healthcare for the automated production of fluorine-18 radiotracers such as [¹⁸F]FDG. The Sep-Pak cartridges used (Accell Plus QMA Carbonate Plus Light Cartridges (130 mg, 37-55 µm; Waters), ¹C18 Plus Short Cartridges (400 mg, 37-55 µm; preconditioned beforehand with ACN (6 mL) and then water (8 mL); Waters), and Oasis® HLB Plus Short Cartridges (225 mg, 60 µm; preconditioned beforehand with EtOH (6 mL) and then water (6 mL)) were from Waters®. ¹⁸O-enriched water was purchased from Cambridge Isotope Laboratories. No-carrier-added [¹⁸F]fluoride was produced via the ¹⁸O(p,n)¹⁸F nuclear reaction on ¹⁸O-enriched water (> 95%) with a Cyclone 18/9 from IBA. At the end of bombardment (EOB), the activity was transferred to the hot lab cell with helium pressure through Teflon tubing (~50 m).

2.2.1. Fully automated radiosynthesis of 1-(2-[¹⁸F]fluoroethyl)-DL-tryptophan (DL-[¹⁸F]5)

The whole radiosynthesis of 1-(2-[¹⁸F]fluoroethyl)-DL-tryptophan was carried out on a FASTlab™ synthesizer from GE Healthcare. The reagents and solvents used for the 1-[¹⁸F]FE-DL-Trp radiosynthesis were placed in small sealed vials. Reagents were prepared and positioned on the FASTlab™ manifold as described in Table 1 and illustrated in Figure 1.

The [¹⁸F]fluoride recovered from the cyclotron (V6) was trapped on an ion exchange resin (QMA Carbonate Cartridge; from V5 to V4) and the [¹⁸O]H₂O was recovered in a separate vial (V1) (Figure 1). The activity was eluted into the reactor through its central tubing (V8) with 750 µL of a Kryptofix® (K_{2.2.2}, 37.5 mg in 600 µL of MeCN) and K₂CO₃ (7.5 mg in 150 µL of water) solution. Water was azeotropically evaporated under vacuum and nitrogen flow by heating at 105°C and 120°C. Then, 1.0 mL of *tert*-butoxycarbonyl-(1-(2-tosyloxyethyl)tryptophan) *tert*-butyl ester (**7**) in anhydrous DMF (9 mg; 0.014 mmol; 1.1 mL) was added to the dry potassium [¹⁸F]fluoride/K_{2.2.2} complex (typically 35-75 GBq) through the central tubing of the reactor. After labeling for 5 minutes at 140°C, the reaction medium containing [¹⁸F]**4** was diluted three times in syringe S2 (V11) with NaCl 0.9% (~ 12 mL), and the *tert*-butoxycarbonyl-(1-(2-[¹⁸F]fluoroethyl)tryptophan) *tert*-butyl ester, [¹⁸F]**4**, was trapped on a ¹C18 cartridge (from V17 to V18). The reactor was washed with NaCl 0.9% (~ 3 mL), and this solution was also passed through the cartridge. A small volume of hydrobromic acid (500 µL, 6N) was transferred into the reactor (syringe S2, V11), and the ¹C18 cartridge was eluted (from V18 to V17; also call as a reverse flow elution) with ethanol (2 mL; syringe S3, V24). The intermediate [¹⁸F]**4** was recovered into the reactor via its central tubing (V8), and the solution was concentrated by heating at 125°C for 6 minutes under a low nitrogen flow. Thereafter, an additional volume of hydrobromic acid (2 mL, 6N; syringe S2) was added and hydrolysis was conducted for ten additional minutes at 90°C. The crude product DL-[¹⁸F]**5** was diluted with NaCl 0.9% (2 mL), and transferred with syringe S2 (V11) into the semi-preparative HPLC loop (V9; 6 mL) through a Sterifix® Paed filter (with a 0.2 µm Supor® membrane). The reactor was then washed with NaCl 0.9% (1.5 mL), and this solution was also transferred into the semi-preparative HPLC loop. The semi-preparative HPLC was performed in *condition*

A and the DL-[¹⁸F]5 HPLC peak was collected after 25 minutes in a sealed vial containing water (21 mL). Once homogenized with nitrogen for 20 seconds, the solution was pumped (from V10), 6 mL by 6 mL, with the syringe S2 (V11) and subsequently passed through two preconditioned Oasis® HLB cartridges (12 mL.min⁻¹; V20 – V21 – V22 – V23). Then, the HLBs were washed with NaCl 0.9% (6 mL). Finally, DL-[¹⁸F]5 was eluted into the outlet vial (V19) with a reverse flow of EtOH (2 mL, syringe S3 (V24)) followed by NaCl 0.9% (2 mL, syringe S3 (V24)), and a slight nitrogen flow. For the chiral HPLC separation of its two enantiomers, DL-[¹⁸F]5, eluted from the HLB SPE cartridge with EtOH (2 mL), was not subsequently diluted with the aqueous solution of NaCl 0.9%.

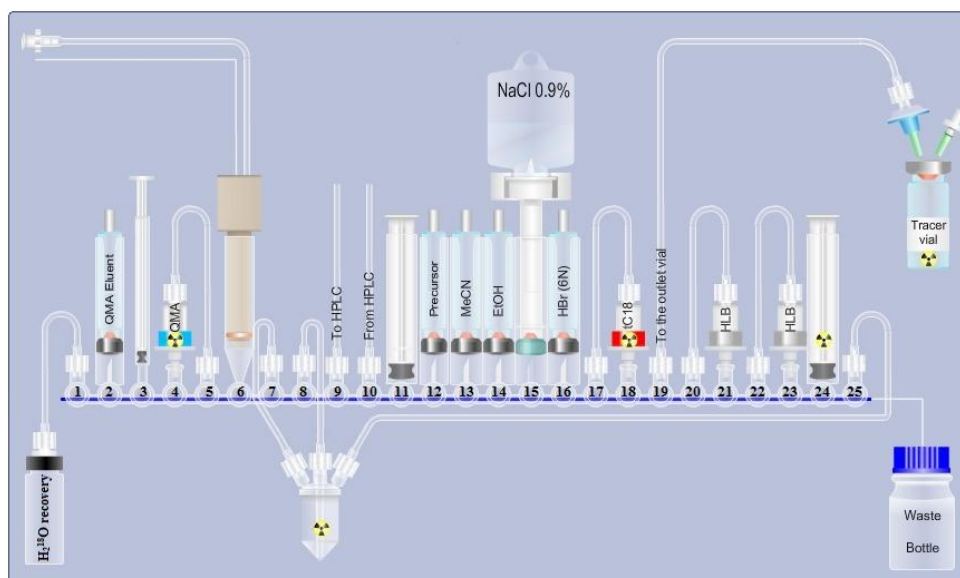


Figure 1. Template of the cassette for the radiosynthesis of 1-[¹⁸F]FE-DL-Trp (DL-[¹⁸F]5) on the FASTlab™ module.

Table 1. Reagents for the radiosynthesis of 1-[¹⁸F]FE-DL-Trp on a FASTlab™ synthesizer.

FASTlab™ manifold valves	Reagent, solvent or SPE cartridge	Details
V1	Silicone tubing to H ₂ ¹⁸ O recovery vial	14 cm
V2	K _{2.2.2.} ® (37.5 mg) in MeCN (600 µL) and K ₂ CO ₃ (7.5 mg) in H ₂ O (150 µL)	11 mm vial (eluent volume: 750µL)
V3	Syringe S1 (part of the manifold)	Maximum 1 mL
V4	Sep-Pak QMA Carbonate Cartridge with silicone tubing at position 5	46 mg (37-55 µm) (Waters)
V5	Silicone tubing to QMA SPE cartridge at position 4	14 cm
V6	H ₂ ¹⁸ O/ ¹⁸ F inlet reservoir (part of the manifold)	Maximum 5 mL
V7	Silicone tubing to the reactor vessel (left-hand side)	14 cm
V8	Silicone tubing to the reactor vessel (central port)	14 cm
V9	Outlet “to HPLC” loop via silicone tubing connected to a Sterifix® Paed filter (B. Braun)	80 – 150 cm
V10	Inlet “from HPLC” allowing the radiotracer recovery after semi-preparative HPLC purification	80 cm
V11	Syringe S2 (part of the manifold)	Maximum 6 mL
V12	Protected tosylated precursor 7 (9 mg) in anhydrous DMF (1.1 mL)	11 mm vial
V13	Acetonitrile	13 mm vial (MeCN volume: 4 mL)
V14	Ethanol	13 mm vial (EtOH volume: 4 mL)
V15	NaCl 0.9% bag spike	Solution volume: 100 mL
V16	Hydrobromic acid (6N)	13 mm vial (HBr volume: 4 mL)
V17	Silicone tubing to Sep-Pak ¹ C18 cartridge at position 18	14 cm
V18	Sep-Pak ¹ C18 Plus Short Cartridge, with silicone tubing at position 17	400 mg (37-55 µm) (Waters)
V19	Final outlet tracer vial	21 cm
V20	Silicone tubing to Oasis® HLB Cartridge (1/2) at position 21	14 cm
V21	Oasis® HLB Plus Short Cartridges (1/2), with silicone tubing at position 20	225 mg (60 µm) (Waters)
V22	Silicone tubing to Oasis® HLB Cartridge (2/2) at position 23	14 cm
V23	Oasis® HLB Plus Short Cartridges (2/2), with silicone tubing at position 22	225 mg (60 µm) (Waters)
V24	Syringe S3 (part of the manifold)	Maximum 6 mL
V25	Silicone tubing to the reactor vessel (right-hand side) and vent valve for the reactor	42 cm

2.2.2. Resolution of the D- and L-enantiomers: 1-[¹⁸F]FE-L-Trp (L-[¹⁸F]5) and 1-[¹⁸F]FE-D-Trp (D-[¹⁸F]5)

An aliquot (500 µL) of the undiluted ethanol solution of 1-[¹⁸F]FE-DL-Trp was resolved on a chiral HPLC column (*conditions B*). The two enantiomers, eluted at 14 – 16 min and 20 – 22 min, respectively, were collected separately in two vials, each of them containing water (21 mL). The separation was repeated once more and the two radioactive peaks were collected again in the same D- and L-vials. The D- and L-enantiomers (D-[¹⁸F]5 and L-[¹⁸F]5) were then formulated onto a second FASTlab™ cassette (Figure 2).

2.2.3. Fully automated formulation of L-[¹⁸F]5 and D-[¹⁸F]5

Once homogenized with a slight nitrogen flow (V23) for 15 seconds (Figure 2 and Table 2), the L-[¹⁸F]5 isomer solution was aspirated, 6 mL by 6 mL, with S3 (V24), and passed very slowly (6 mL.min⁻¹) through an Oasis® HLB cartridge (from V19 to V18). Then, the HLB cartridge was washed with a HOAc/NaOAc buffer solution (6 mL; pH 4). Finally, L-[¹⁸F]5 was eluted (from V18 to V19) with a reverse flow of EtOH (2 x 1 mL (V13); syringe S1 (V3)), followed by NaCl 0.9% (2 mL; syringe S1 (V3)), and a slight nitrogen flow towards the outlet – “L” tracer vial (V22).

The other isomer (D-[¹⁸F]5) was also formulated on the same FASTlab™ cassette (Figure 2 and Table 2). After homogenization with nitrogen (V10) for 15 seconds, the D-[¹⁸F]5 containing solution was pumped, 6 mL by 6 mL, with S2 (V11) and passed through another Oasis® HLB cartridge (from V5 to V4; 6 mL.min⁻¹). The HLB cartridge was washed with a HOAc/NaOAc buffer solution (6 mL; pH 4). D-[¹⁸F]5 was subsequently eluted (from V4 to V5) towards the outlet – “D” tracer vial (V9) with a reverse flow of EtOH (V12 (1 mL) and V2 (1 mL); syringe S1 (V3)), followed by NaCl 0.9% (2 mL; syringe S1 (V3)), and a slight nitrogen flow.

2.2.4. Quality control and stability studies of DL-[¹⁸F]5, L-[¹⁸F]5, and D-[¹⁸F]5

Radiochemical purity was determined after analytical UPLC analyses (*conditions C and D*), chiral HPLC analyses (*condition B*), and radioTLC (R_f: 0.60 (*condition F*)). The radiochemical stability was checked up to 4 h after the end of synthesis (EOS) at room temperature (n = 7).

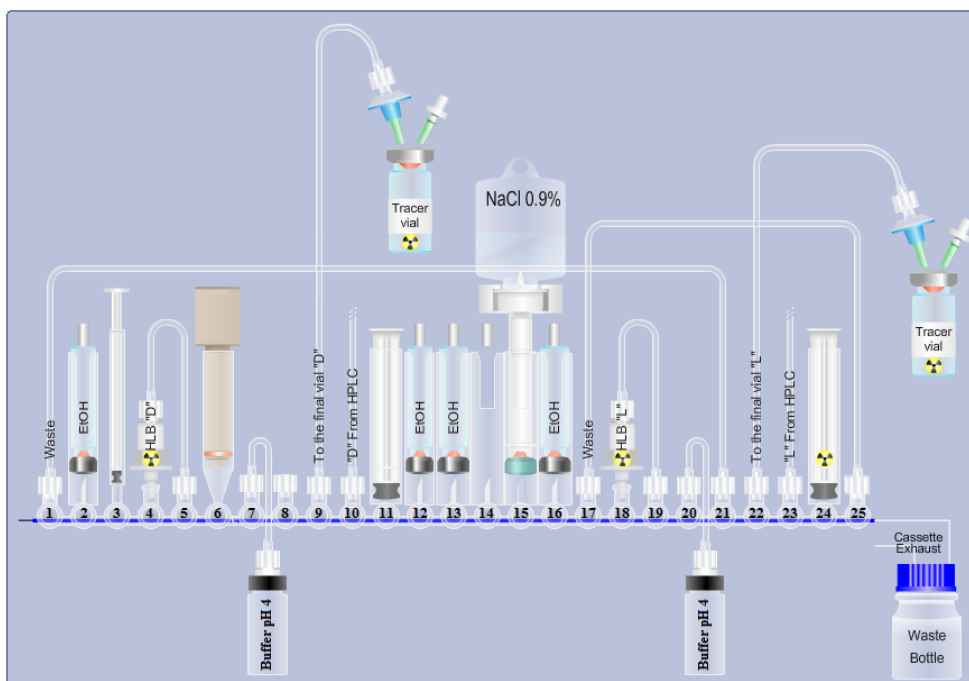


Figure 2. Template of the cassette for the formulation of 1-[¹⁸F]FE-L-Trp (L-[¹⁸F]5) and 1-[¹⁸F]FE-D-Trp (D-[¹⁸F]5) on the FASTlab™ module.

Table 2. Reagents for the formulation of 1-[¹⁸F]FE-L-Trp and 1-[¹⁸F]FE-D-Trp on a FASTlab™ synthesizer.

FASTlab™ manifold position		Reagent, solvent or SPE cartridge
“D-isomer” Formulation	V2	EtOH (2.5 mL)
	V4	Oasis® HLB Plus Short Cartridge (225 mg (60 µm), Waters)
	V7	HOAc/NaOAc buffer solution (100 mM; pH 4; 4 mL)
	V9	Outlet - “D” tracer vial
	V10	Tubing “from HPLC” allowing the recovery of D-[¹⁸ F]5 after chiral HPLC separation
	V13	EtOH (4 mL)
	V15	Spike for NaCl 0.9% bag (100 mL)
V1-V21		Waste loop
“L-isomer” Formulation	V12	EtOH (2.5 mL)
	V15	Spike for NaCl 0.9% bag (100 mL)
	V16	EtOH (4 mL)
	V18	Oasis® HLB Plus Short Cartridge (225 mg (60 µm), Waters)
	V20	HOAc/NaOAc buffer solution (100 mM; pH 4; 4 mL)
	V22	Outlet - “D” tracer vial
	V23	Tubing “from HPLC” allowing the recovery of L-[¹⁸ F]5 after chiral HPLC separation
V17-V25		Waste loop

2.2.5. Determination of lipophilicity (logP and logD) of DL-[¹⁸F]5

Partition coefficient (*log P*; *n*-octanol/water): DL-[¹⁸F]5 (~37 MBq; 50 µL) was added to an Eppendorf cap containing *n*-octanol (500 µL) and water (450 µL). The vial was strongly vortexed (15 min) and then centrifuged at 14600 rpm (3 min). An aliquot of each layer (150 µL) was taken off and assessed for radioactivity. Distribution coefficient (*log D*; *n*-octanol/buffer pH 7.4): the procedure described above was followed with 0.1M sodium phosphate buffer (450 µL; pH 7.4) instead of water. *Log P* and *log D* 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, as reported by Damont A. & al.[11]

2.3. Radiopharmacology

2.3.1. Cell cultures – Cell lines

P815B is a subline of mastocytoma P815 used as a vector-transfected control.[1] P815B cells were transfected with an expression vector pEF2 containing either the mouse or human *Indo* ORF or the mouse or human *TDO* ORF.[1,12] Cells selected for the experiments were P815B clone 1 (control cells, no expression of IDO or TDO), P815B-mIDO clone 6 and P815B-hIDO clone 6 (high expression of either mouse [m] or human [h] IDO), P815B-mTDO clone 12 or P815-hTDO clone 19 (high expression of TDO). Cells were cultured in IMDM (Iscove's Modified Dulbecco's Medium with Glutamine and Hepes) supplemented with 10% FCS (fetal calf serum) at 37°C in a humidified incubator with 5% CO₂.

2.3.2. In vitro cellular uptake studies with DL-[¹⁸F]5, L-[¹⁸F]5 and D-[¹⁸F]5

Cells were washed three times with HBSS buffer (Hank's Balanced Salt Solution; centrifugation between each washing: 200g, 5 minutes) and preincubated in HBSS supplemented or not with 10 µM L-tryptophan for one hour at 37°C. A total of 2 million cells were used in each experimental tube. Around 3.7 MBq of 1-[¹⁸F]-FETrp were added to the cell suspension. 1-Methyl-L-tryptophan (1-L-MT, 400 µM), an inhibitor of IDO, was eventually added in the incubation medium of P815B-mIDO cl6 (at the same time as the radiotracer). After 1 to 60 minutes of incubation at 37°C under an atmosphere of 5% CO₂, cells were put on ice, centrifuged at 200g for 5 minutes and then, washed three times with PBS (phosphate-buffered saline). Pellets and supernatants were counted in a gamma counter (Cobra - Autogamma) in order to estimate the tracer uptake expressed as a percentage of the total activity added to the cell suspension.

To estimate the retention rate of the labeling, cells were again suspended in HBSS containing or not 10 μ M L-tryptophan and maintained at 37°C for 1 hour. The cell suspension was then centrifuged at 150g for 5 min and the pellet separated from the supernatant. The tracer efflux, expressed in % of the incorporated activity, was estimated from the activity counted in the pellet and in the medium.

3. Results and discussion

3.1. Chemistry

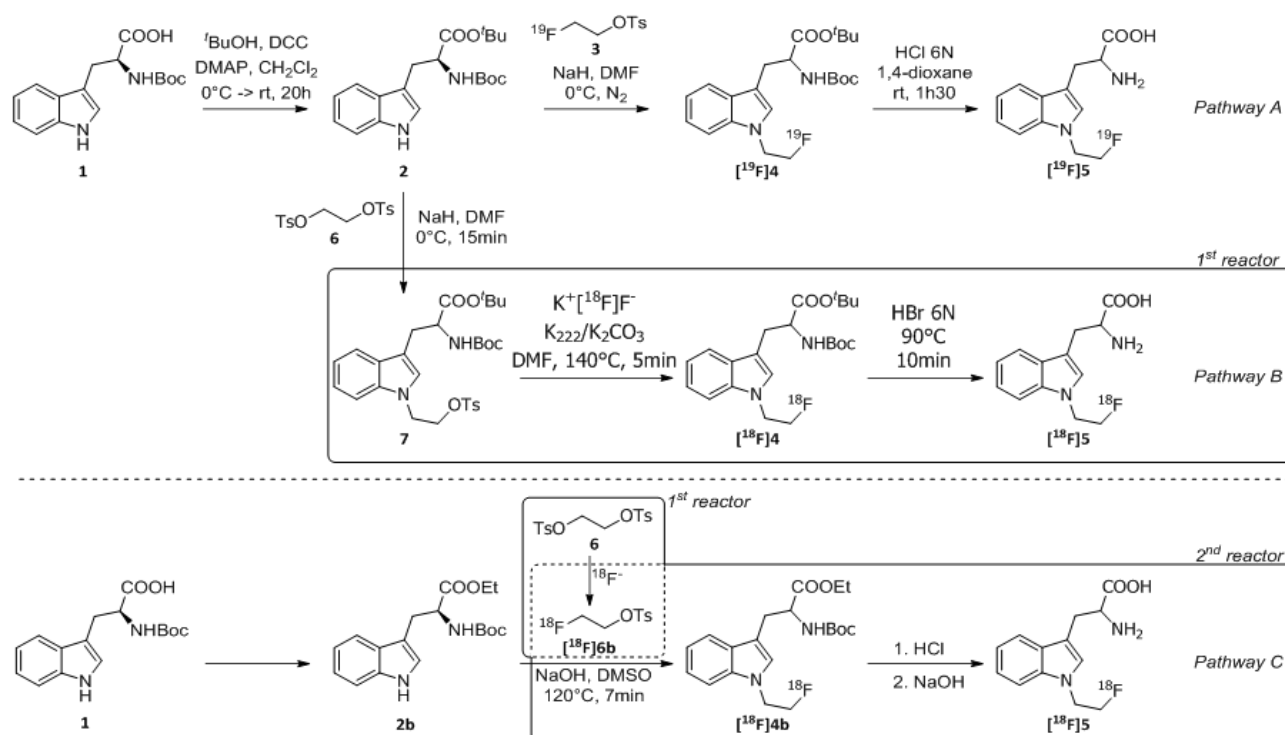
Recently, Sun et al. have described a three-step (two-pots) radiosynthesis of [18 F]**5**.^[9] This method (Scheme 1, Pathway C) involved the prior radiosynthesis of a small prosthetic group [18 F]**6b** (by the mono-labeling of a 1,2-ditosyloxyethane, **6**), which was used for the alkylation of **2b** in basic media. After removing the protecting groups of [18 F]**4b**, [18 F]**5** was obtained with a radiochemical yield (n.d.c.) of $0.97 \pm 0.2\%$ (2.7 ± 0.6 MBq; n = 5; in 65 minutes; radiochemical purity: 95-97 %).^[9] Moreover, even though the basic conditions used for the alkylation could induce an epimerization of the product, no values of the enantiomeric excess (e.e.) are mentioned by these authors.

Due to the drawbacks of this method, we developed a new strategy for the radiosynthesis of this molecule. Our two-steps (one-pot) radiosynthesis of [18 F]**5** implied the fast, regioselective and direct radiofluorination of the protected tosylate precursor **7** (synthesized beforehand from 1,2-ditosyloxyethane (**6**) and **2** in basic media), which is, from a strategic point of view, more preferable than a multistep approach. After labeling, the hydrolysis of the protecting groups of the intermediate [18 F]**4** was performed in acidic conditions (Scheme 1, Pathway B). This synthesis was implemented and improved on a FASTlabTM automated synthesizer (single used cassette, COC reactor, open access software) (Figure 1 and Table 1).

3.1.1. Synthesis of *tert*-butoxycarbonyl-(1-(2-tosyloxyethyl)tryptophan) *tert*-butyl ester (**7**)

As shown in Scheme 1, the first step of our strategy involved the protection of the L-tryptophan with a *tert*-butoxycarbonyl (Boc) and a *tert*-butyl ester, both protecting groups being stable in basic media and easily removable in acidic conditions. This key intermediate **2** was used for the syntheses of the [19 F]fluoro-references (*tert*-butoxycarbonyl-(1-(2-[19 F]fluoroethyl)tryptophan) *tert*-butyl ester ([19 F]**4**) and 1-(2-[19 F]fluoroethyl)-DL-tryptophan (DL-[19 F]**5**)^[5] (Scheme 1, Pathway A)), and those of the starting tosylate precursor **7** required for the radiosynthesis (Scheme 1, Pathway B).

The *tert*-butoxycarbonyl-(1-(2-tosyloxyethyl)tryptophan) *tert*-butyl ester **7** was obtained in only one step from **2** (Scheme 1). In order to minimize side reactions such as potential intermolecular dialkylation or deprotection, the alkylation on the nitrogen atom of the indole ring was conducted under several conditions (Table 3) and the conversion was followed by analytical HPLC analyses. With relatively low amounts of base and alkylating agent (from 1.5 to 6 equiv.), only poor and unsatisfactory conversions were observed after a short reaction time (Table 3; entries 1 & 3). In the conditions of entries 2 and 4, a complete consumption of reactant **2**, with an increased conversion rate to the detriment of its purity, was observed. In fact, when the duration of the alkylation was extended, the formation of numerous side products was highly favored, making the purification of **7** more difficult. On the other hand, the best conversions were obtained when the alkylation was carried out for only 15 min in a DMF solution containing a very large excess of 1,2-ditosyloxyethane **6** (10 equiv.; favoring the alkylation with respect to the intermolecular dialkylation) and sodium hydride (6 equiv) (entries 5). In these conditions, side reactions were minimized, and **7** was formed as the major product, facilitating therefore its purification. The tosylate precursor was thus recovered after HPLC purification with a yield of 49% (n = 3), but with a complete racemization. Nevertheless, as our previous studies have demonstrated that 1-[19 F]FE-DL-Trp is a good and specific substrate of rhIDO,^[5] the racemization of the precursor during its synthesis should not be considered as a drawback. Furthermore, if the *in vitro* cellular uptake of DL-[18 F]**5** confirmed those obtained with its cold reference, this radiosynthesis followed by a chiral HPLC separation should allow the recovery of both resolved [18 F]labeled enantiomers which might be subject individually to further *in vitro* cellular studies.



Scheme 1. General scheme for the synthesis of 1-(2-[¹⁹F]fluoroethyl)-tryptophan (DL-[¹⁹F]5) (Pathway A), the radiosynthesis of 1-(2-[¹⁸F]fluoroethyl)-tryptophan (DL-[¹⁸F]5) following our strategy (Pathway B), or following the strategy involved by Sun & al. (Pathway C). [9] Reactions carried out in the same reactor are framed together in the pathways above.

Table 3. Precursor 7 synthesis - Alkylation of Boc-L-tryptophan *tert*-butyl ester 2 with 1,2-ditosyloxyethane 6 at 0°C.

Entry	6 (equiv.)	Base (equiv.)	Time	Conversion (Yield)
1	1.5	NaH (1.5)	15 min	< 1% ^a
2			4 h	~20% ^b
3	6	NaH (6)	15 min	< 5% ^a
4			3 h	~21% ^b
5	10	NaH (6)	15 min	53-58% ^c (49%) ^d

^a Only low conversion observed; ^b Limited conversion observed due to the formation of numerous side products; ^c Higher conversion observed due to limited side products formation; ^d The yield was determined after semi-preparative HPLC (n = 3).

3.2. Radiochemistry

3.2.1. Fully automated n.c.a. radiosynthesis of 1-[¹⁸F]FETrp (DL-[¹⁸F]5) on FASTlabTM synthesizer

Preliminary tested manually, the two steps of the n.c.a. radiosynthesis of DL-[¹⁸F]5 were then implemented and optimized on a FASTlabTM synthesizer. In fact, in addition to its reliability, this remote-controlled device allows highly reproducible radiosyntheses (*e.g.* same temperature in the reactor, same elution speed, same nitrogen flow, same step duration), while reducing the exposure of the operator, which is ideal, both for the development of the radiosynthesis and for further routine production of the radiopharmaceutical compound. The labeling was carried out with [¹⁸F]fluoride previously activated by potassium carbonate and Kryptofix[®] (K_{2.2.2}). [13] The influence of the labeling parameters (solvent, duration, and temperature) on the radiochemical decay-corrected yield (RCY) obtained for the radiofluorination of 7 was evaluated by TLC (*condition E*). According to the data presented in Table 4, the highest RCY (57%, decay-corrected) was obtained in DMF (5 minutes; 140°C; n = 3). In the same conditions, acetonitrile (Bp: 82°C) was lost by evaporation, leading to the degradation of the [¹⁸F]labeled compounds. At lower temperature (90°C), this evaporation was reduced. However, the RCYs were always lower, even after 10 minutes.

Table 4. Labeling of the Boc-(1-(2-tosyloxyethyl)tryptophan) *tert*-butyl ester 7 – [¹⁸F]4 synthesis.

Solvent	Temperature	Duration	RCY ^a
CH ₃ CN (Bp: 82°C)	90 °C	3 min	25%
	120 °C	10 min	28%
	140 °C	5 min	nd ^b
DMF (Bp: 152°C)	95 °C	5 min	11%
	140 °C	3 min	51%
	140 °C	5 min	57% (n = 3)

^a RCY: Radiochemical decay-corrected yields; ^b nd: no determined due to solvent evaporation causing product degradation.

After labeling, the crude reaction mixture was diluted with water and DL-[¹⁸F]4 was trapped on a ¹C₁₈ SPE cartridge. During this process, [¹⁸F]fluoride, K_{2.2.2}, K₂CO₃ and DMF are eliminated. Elution of the SPE cartridge with EtOH offered pre-purified DL-[¹⁸F]4. Addition of hydrobromic acid (6 N) before ethanol evaporation (6 min) avoids the degradation and the loss of the [¹⁸F]labeled compound once the ethanol is evaporated. The hydrolysis was then extended at lower temperature (90°C, 10 minutes) with two supplementary milliliters of HBr (6N) (radiochemical purity: 75 ± 2%, n = 3). This step was also tested with hydrochloric acid (several concentrations and duration), but the removal of the protecting groups was always less effective than with HBr. In a typical experiment, labeling and hydrolysis take around 42 minutes.

Purification of the crude DL-[¹⁸F]5 was implemented on a semi-preparative HPLC column. As mentioned by Lemaire and co-workers, the injection is not influenced by the length of the silicone tubing (between the synthesizer and the HPLC loop) as a hydrophilic/hydrophobic filter is placed at the entrance of the HPLC loop (Table 1).[10] After 20 minutes, the radioactive peak of DL-[¹⁸F]5 was collected and formulated on the same FASTlabTM cassette (Figure 1). This formulation needed two HLB SPE cartridges to trap DL-[¹⁸F]5 quantitatively. The cartridges were then washed with NaCl 0.9%, and eluted successively with a reverse flow of EtOH and NaCl 0.9%.

The fully automated process (labeling, hydrolysis, semi-preparative HPLC, and formulation) offered DL-[¹⁸F]5 within 80 minutes with a global radiochemical decay-corrected yield of 30 ± 4% (n = 7). Starting from 65 – 70 GBq (EOB) of [¹⁸F]fluorides, more than 12.7 ± 3.5 GBq (n = 7) of DL-[¹⁸F]5 were obtained at the end of synthesis (EOS), with a radiochemical purity over 98% and a specific activity determined one hour after the formulation of 600 ± 180 GBq/μmol (n = 5).

RadioTLC and UPLC quality controls (Figures 3A and 3B) performed on the final solution (50 – 75 mCi/mL; EtOH/NaCl 0.9% (1/1)) show none radiolysis of DL-[¹⁸F]5 even 4h after its formulation. Just before cellular *in vitro* studies, this solution was diluted with NaCl 0.9% to provide an injectable aqueous solution of 1-[¹⁸F]FE-DL-Trp containing 1 – 5 % of ethanol.

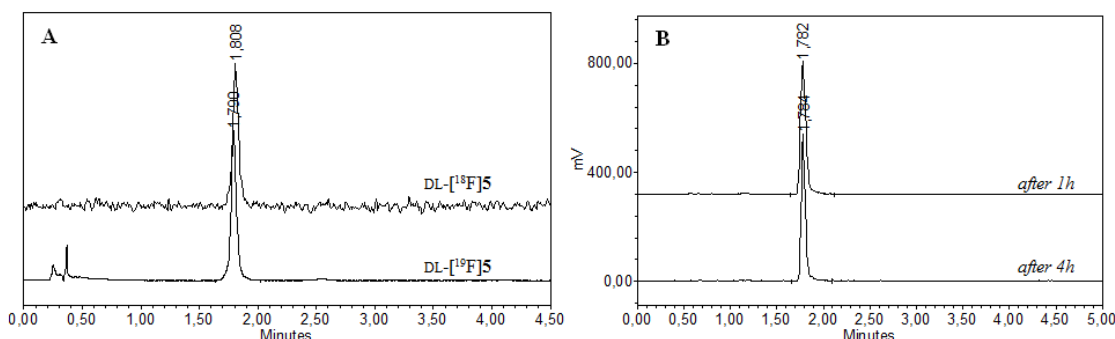


Figure 3. UPLC Quality Control: A. coinjection of DL-[¹⁸F]5 (chromatogram above) and its cold reference (chromatogram below), B. (radio)stability of DL-[¹⁸F]5 (chromatogram above: 1h after the formulation; chromatogram below: 4h after the formulation).

3.2.2. Chiral separation and fully automated formulation of L-[¹⁸F]5 and D-[¹⁸F]5 on FASTlabTM synthesizer

Since DL-[¹⁸F]5 was obtained as a racemate, its two [¹⁸F]labeled enantiomers (D-[¹⁸F]5 and L-[¹⁸F]5) were thus recovered after their subsequent resolution performed twice on an analytical chiral HPLC column (*condition B*). By this way, the affinity and specificity of these three potential radiotracers for IDO-expressing cells could be determined and compared. An important parameter to note is the high performance of this analytical HPLC column. In fact, even though the volume injected is high (500 μ L per injection) as compared to the elution flow rate (700 μ L.min⁻¹), the chiral resolution of the peaks still remains very good (R_S (100 μ L): 2.01 (Figure 4A) and R_S (500 μ L): 2.16 (Figure 4B); Δt_R : 6 min; Figures 4A and 4B), and the peaks baseline width (W_b about 3 min) does not increase (with respect to smaller volumes, Figures 4A (100 μ L; W_b : 3.00 min (L) and 3.05 min (D)), 4B (500 μ L; W_b : 2.86 min (L) and 3.33 min (D)), 4C (150 μ L), and 4D (200 μ L)). This could be explained by the fact that the injected mass of product is very small (n.c.a. radiosynthesis approach), and because the injected sample (DL-[¹⁸F]5 in EtOH) and the HPLC eluent (MeOH/H₂O 85/15 (v/v)) have similar matrices. As only small amounts of activity were required for the first *in vitro* cellular uptake experiments, only half of the DL-[¹⁸F]5 solution was resolved by two successive chiral HPLC separations of the [¹⁸F]labeled enantiomers.

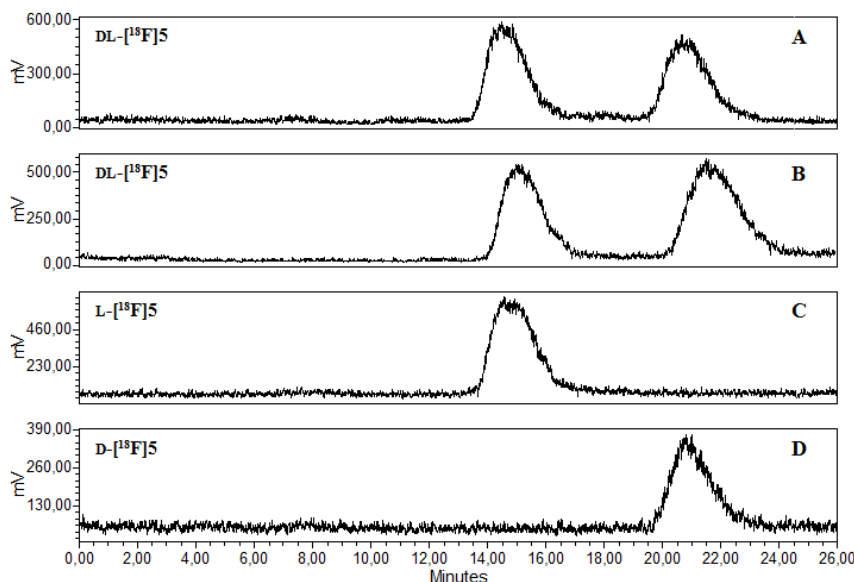


Figure 4. Chiral HPLC chromatograms on an Astec ChirobioticTM T column (*condition B*).

A. Volume injected: 100 μ L of DL-[¹⁸F]5 (after formulation) – peaks baseline width (W_b): 3.00 min (L) and 3.05 min (D); B. Volume injected: 500 μ L of DL-[¹⁸F]5 (after formulation) – W_b : 2.86 min (L) and 3.33 min (D); C. Volume injected: 150 μ L of L-[¹⁸F]5 (reinjecting after chiral separation and formulation) – W_b : 3.00 min; and D. Volume injected: 200 μ L of D-[¹⁸F]5 (reinjecting after chiral separation and formulation) – W_b : 3.08 min. The volume injected on the HPLC column corresponds to that of the loop.

The resolved D- and L-enantiomers were collected in two separated vials. After dilution with water, they were formulated on a second FASTlabTM cassette (Figure 2). The right part of the cassette was dedicated to the formulation of the L-isomer (the first HPLC peak collected) and the left part to the D-isomer (the second peak collected). With these settings, as the nitrogen flow is always directed from the left to the right, the tubing of the left part remain clean and dry throughout the formulation of the L-[¹⁸F]5, and until that of the D-[¹⁸F]5, minimizing therefore the potential contamination between both enantiomers (as confirmed by the reinjection of the formulated L-[¹⁸F]5 and D-[¹⁸F]5; Figures 4C and 4D).

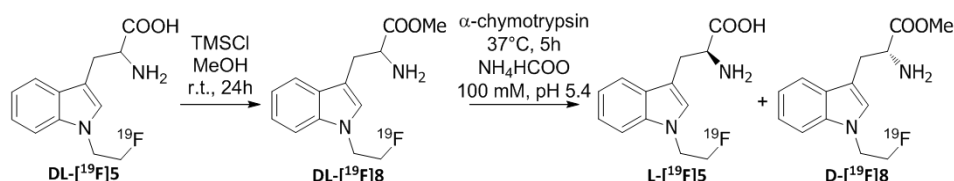
The formulation of the enantiomers required the use of pre-conditioned HLB SPE cartridges to trap L-[¹⁸F]5 (or D-[¹⁸F]5) quantitatively. The first experiments, conducted with a trapping flow rate of 12 mL.min⁻¹ (as previously described for the formulation of the racemate), required two HLB SPE cartridges (225 mg) to trap quantitatively each resolved isomer, but raising significantly the cost of such formulation. As a consequence, the trapping was improved by decreasing the flow rate to 6

mL.min⁻¹, and allowing thereby the use of only one HLB SPE cartridge per isomer. Several elution conditions were also tested (Table 5). Even though the trapping occurs at pH 6.5, quantitative elution with a reverse flow of ethanol is only possible if the cartridge has been washed beforehand with a HOAc/NaOAc buffer solution (2 mL, 100 mM) at pH 4 (Table 5, entry 4). L-[¹⁸F]5 and D-[¹⁸F]5 were recovered following this procedure with a RCY for their respective formulation of 96% (n = 2, d.c.) and 92% (n = 2, d.c.), and with an overall RCY of 6.1% (d.c.; in 155 minutes from EOB; n = 2; 1.6 – 1.8 GBq range) and 5.8% (d.c.; in 180 minutes from EOB; n = 2; 1.3 – 1.6 GBq range), respectively. The chemical and radiochemical purities of the enantiomers, and the stability of these compounds up to 4h after their formulation were checked by chiral HPLC (Figures 4C and 4D) and radioTLC analyses. An injectable aqueous solution of 1-[¹⁸F]FE-L-Trp (or 1-[¹⁸F]FE-D-Trp, respectively) containing 1 – 5 % of EtOH was formed by dilution with aqueous NaCl 0.9% just before its use in cellular *in vitro* assays.

Table 5. HLB cartridges elution tests for the formulation of L-[¹⁸F]5 and D-[¹⁸F]5.

Entry	Procedure and conditions for HLB SPE cartridges elution	Elution yield
1.	1. washing with NaCl 0.9% in aqueous solution 2. reverse flow elution with EtOH (2 mL)	47%
2.	1. washing with NaCl 0.9% in aqueous solution 2. reverse flow elution with EtOH (2 mL) 3. reverse flow elution with NaCl 0.9% (2 mL)	66%
3.	1. washing with 2 mL of HOAc/NaOAc buffer (pH 4, 100 mM) 2. reverse flow elution with EtOH (2 mL)	80%
4.	1. washing with 2 mL of HOAc/NaOAc buffer (pH 4, 100 mM) 2. reverse flow elution with EtOH (2 x 1 mL) 3. reverse flow elution with NaCl 0.9% (2 x 1 mL)	95 ± 3 % (n = 5)

The stereochemistry attribution of both [¹⁸F]labeled products resolved on the chiral HPLC column was proved by the enzymatic resolution of 1-(2-[¹⁹F]fluoroethyl)-DL-tryptophan methyl ester, DL-[¹⁹F]8, treated with crystalline α -chymotrypsin. After an enzymatic treatment with this esterase, which is stereospecific to the L-amino acid,[14–16] only the L-enantiomer must be cleaved (Scheme 2). A part of the resulting crude reaction mixture was analyzed on analytical RP18 HPLC. The HPLC peak corresponding to the L-hydrolyzed product was collected and reinjected into the chiral HPLC column (condition B). This analysis confirms that the first peak appearing in this chiral chromatogram corresponds to L-5. The stereospecificity of α -chymotrypsin for the L-amino acids was also assessed with L-tryptophan methyl ester (complete hydrolysis observed), D-tryptophan methyl ester (no reaction observed), and epimerized (L/D 75/25(w/w)) tryptophan methyl ester (75% of hydrolysis observed).



Scheme 2. Enantioselective synthesis of L-[¹⁹F]5 with α -chymotrypsin used for the attribution of the chiral HPLC peaks.

3.3. Determination of hydrophilicity (log P and log D) of DL-[¹⁸F]5

Log P (n-octanol/water) and log D (n-octanol/buffer pH 7.4) of DL-[¹⁸F]5 were determined as described above by the shake flask method. Values of -0.59 and -0.70 were found respectively (n = 2). These data confirmed the hydrophilic nature of the amino acid DL-[¹⁸F]5.

3.4. In vitro enzymatic assays of D-[¹⁸F]5 and L-[¹⁸F]5 with rhIDO: qualitative tests

In order to compare the affinity of both enantiomers for rhIDO, some *in vitro* enzymatic assays were firstly performed with D-[¹⁸F]5 and L-[¹⁸F]5. As described in Table 6, L-[¹⁸F]5 is consumed much more rapidly than its D-enantiomer. These first

results suggest a higher affinity of rhIDO for the L-enantiomer, probably due to a better disposition in the cavity of the enzyme, allowing, by this fact, better H-bonding interactions between NH_3^+ group and the catalytic center.[5,17]

Table 6. *In vitro* enzymatic assays of L-[^{18}F]5 and D-[^{18}F]5 with rhIDO.

Substrate ^a	[rhIDO] _{solution} (μM)	Time (min)	Kynurenine derivative formed
L-[^{18}F]5	4.8	10	100%
	4.8	5	100%
	0.96	10	33%
	0.96	5	28%
D-[^{18}F]5	4.8	10	46%
	4.8	5	40%

^a 86 μL of [^{18}F]5 in 5% of EtOH solution (total volume: 100 μL) were added to start the reaction carried out at room temperature.

3.5. *In vitro* cell uptake of L-, D-, and DL-[^{18}F]5

In order to study the affinity and the specificity of these new potential PET tracers of hIDO, and confirm the preliminary *in vitro* enzymatic results previously obtained, *in vitro* cellular uptake was investigated with D-, L- and DL-[^{18}F]5. Figure 5 showed the progressive accumulation of DL-[^{18}F]5 in P815B-mIDO c16 cells expressing high levels of mIDO, over a 60-minute period. The uptake was clearly lower either in P815B-IDO negative c11 cells or in P815B-mTDO c112 cells expressing high levels of mTDO, a non-homologous enzyme of mIDO constitutively expressed in liver, where it acts as the main regulator of systemic tryptophan levels (Figure 5A).[3,6,12] The presence in the incubation medium of 1-L-MT (400 μM), an IDO inhibitor, reduced the uptake of DL-[^{18}F]5 in P815B-mIDO c16 cells to the levels observed in P815B c11 and P815B-mTDO c112 cells, or lower (Figure 5A, green bars). The presence of L-tryptophan during the preincubation period and the incubation with the tracer, markedly decreased the accumulation of DL-[^{18}F]5 in P815B-mIDO c16 cells, but was without notably effect on the uptake levels in P815B c11 and P815B-mTDO c112 cells (Figure 5B). This significant decrease was probably due to a competition between both substrates with a preference for the more active one, L-Trp.

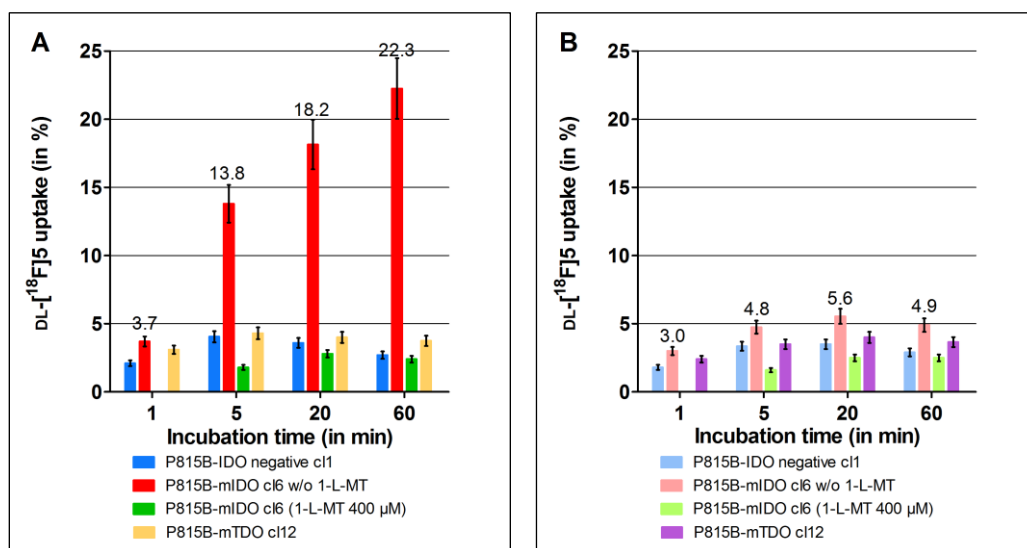


Figure 5. Cellular uptake of DL-[^{18}F]5 (expressed as a percentage of total activity introduced in incubation medium). A. Progressive accumulation of DL-[^{18}F]5 in mIDO expressing cells over a 60-minute period; reduced uptake of DL-[^{18}F]5 in mIDO expressing cells in the presence of 1-L-MT (400 μM). Activity added to the cells: 100-120 μCi (50 μL) (n = 2). B. Accumulation of DL-[^{18}F]5 followed over a period of 60 minutes after preincubation with L-Trp (10 μM). Activity added to the cells: 100-120 μCi (50 μL) (n = 2).

Figure 6 compared the accumulation of racemate, L- and D-enantiomers of 1-[^{18}F]FETrp. The uptake of the D-enantiomer was negligible (non significant) in the three cell lines (Figure 6C). However, as D-[^{18}F]5 was found to be a substrate of hIDO (Table 6), these results suggest a much better transport of the L-enantiomer into the cells. On the other hand, L-[^{18}F]5

was found to present high uptake in hIDO expressing cells and to have a high specificity for this cell line as compared to the two others (hTDO positive cells, and IDO negative cells) (Figure 6B, red bars). Furthermore, as previously described for the racemate (which has a cellular uptake between 30% and 50% of that of the L-enantiomer (Figures 6A and 6B)), the presence in the incubation medium of an inhibitor of hIDO, such as 1-L-MT (400 μ M), also induced a significant decrease in the L- 18 F]radiotracer uptake (Figure 6B, green bars).

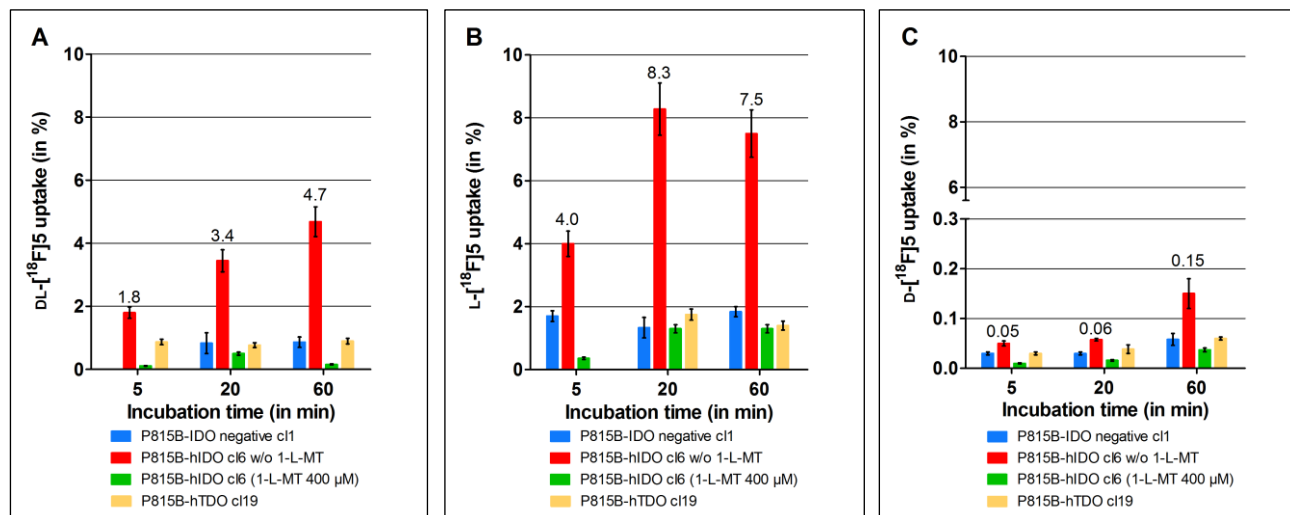


Figure 6. Cellular uptake of DL- 18 F]5, L- 18 F]5, and D- 18 F]5, respectively (expressed as a percentage of total activity introduced in incubation medium). A. Progressive accumulation of DL- 18 F]5 in hIDO expressing cells; reduced uptake of DL- 18 F]5 in hIDO expressing cells in the presence of 1-L-MT (400 μ M). B. Higher progressive accumulation of L- 18 F]5 in hIDO expressing cells; reduced uptake of L- 18 F]5 in hIDO expressing cells in the presence of 1-L-MT (400 μ M). C. Negligible accumulation of D- 18 F]5 (in the presence or not of 1-L-MT (400 μ M)). Activity added to the cells: 80-220 μ Ci (100 μ L; 10% of ethanol) (n = 2).

3.6. In vitro cell efflux of 1- 18 F]FE-DL-Trp (DL- 18 F]5)

In the experimental conditions associated with a high uptake (absence of L-tryptophan and absence of 1-L-MT), the efflux of radioactivity, after a wash out period of one hour, was low in P815B-mIDO cl6 cells. Indeed, the percentage of activity recovered in the supernatant was progressively decreasing for increasing durations of incubation with DL- 18 F]5. As shown in Figure 7, 66 % of the activity accumulated in the cells during a one-minute incubation with DL- 18 F]5 was found in the supernatant, while only 23 %, 21% and 17 % were recovered after 5, 20 and 60 minutes of incubation respectively. This result suggests a slow and progressive oxidation of the radiotracer once in the P815B-mIDO cl6 cells, leading to a tracer trapping favorable to PET imaging.

In the presence of L-tryptophan and/or 1-L-MT, the efflux of the radioactivity from P815B-mIDO cl6 cells was as elevated as in P815B-IDO negative cl1 and in P815B-mTDO cl12 cells, ranging from 73 % to 96 % (Figure 7; pink, green and light green bars). These results can be explained by the competitive effect of L-tryptophan and the inhibitory effect of 1-L-MT on DL- 18 F]5 oxidation by indoleamine 2,3-dioxygenase. As a consequence, the interactions between the PET radiotracer and the enzyme are less effective, favoring therefore the efflux of the radioactivity.

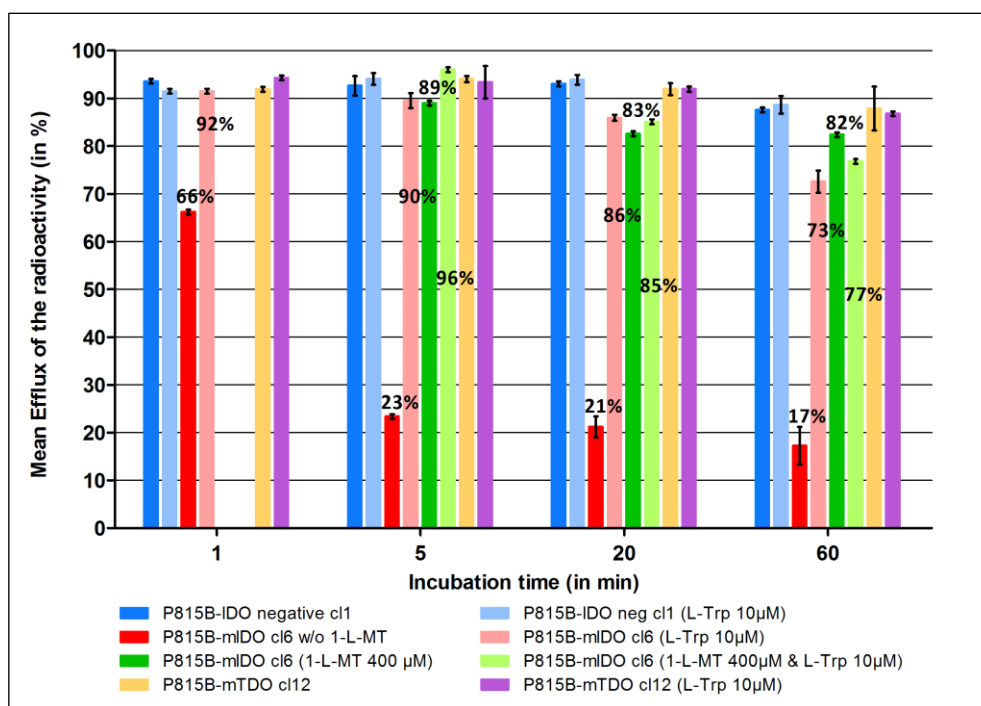


Figure 7. Cellular assays – Efflux of the radioactivity measured after a wash out period of 1h, in function of the time of incubation with DL-[¹⁸F]5 (in the presence or not of 1-L-MT (400 μM), and preincubated with or without L-Trp (10 μM)) (n = 2).

4. Conclusions

In summary, this article describes a simplified and fully automated n.c.a. radiosynthesis of 1-(2-[¹⁸F]-fluoroethyl)-DL-tryptophan designed and implemented on a FASTlabTM module from *GE Healthcare* using single use cassettes. This radiosynthesis offers DL-[¹⁸F]5 with a global radiochemical decay-corrected yield of $30 \pm 4\%$ (radiochemical purity: $>98\%$, n = 7) in only 80 minutes, and with a specific activity of 600 ± 180 GBq/μmol (EOB: 65 – 70 GBq; n = 5). Since a racemate was obtained by this radiosynthesis, the separation of its L- and D- [¹⁸F]labeled enantiomers was conducted on an analytical chiral HPLC column. After their formulation, L-[¹⁸F]5, D-[¹⁸F]5 and DL-[¹⁸F]5 were subjected to enzymatic and cellular *in vitro* tests as potential substrates of hIDO (or mIDO) and hTDO (or mTDO). Among these radiotracers, L-[¹⁸F]5 exhibits the highest specificity and affinity for indoleamine 2,3-dioxygenase expressing cells, as compared to the two other substrates (D-[¹⁸F]5 and DL-[¹⁸F]5), while presenting a very low efflux. These results demonstrate the great interest to develop a stereospecific radiosynthesis of L-[¹⁸F]5 as well as additional *in vivo* cellular imaging studies with these radiotracers. These investigations, both currently under progress in our laboratories, should corroborate these results, and confirm that L-[¹⁸F]5 could be a novel PET imaging agent, potentially very useful i) to improve the detection and the discrimination of hIDO expressing cells in cancer imaging, and ii) to facilitate the development, the preclinical and the clinical validation of new potential inhibitors of human indoleamine 2,3-dioxygenase.

Acknowledgments

The Region Wallonne (Keymarker Project and Cantol Project (BioWin)) is gratefully acknowledged for the financial support provided for this work. Authors also warmly thank Pr. Moreno Galleni, Dr. Frédéric Sapunarc, and Ms Sophie Laurent from the Center for Protein Engineering (ULg, Belgium) for the production and the delivery of the rhIDO enzyme. Alain Plenevaux is research director from FRS-FNRS Belgium.

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