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Development and characterization of a promising fluorine-18 labelled radiopharmaceutical for in vivo imaging of fatty acid amide hydrolase ☆

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ABSTRACT

Fatty acid amide hydrolase (FAAH), the enzyme responsible for terminating signaling by the endocannabinoid anandamide, plays an important role in the endocannabinoid system, and FAAH inhibitors are attractive drugs for pain, addiction, and neurological disorders. The synthesis, radiosynthesis, and evaluation, in vitro and ex vivo in rat, of an ^{18}F-radiotracer designed to image FAAH using positron emission tomography (PET) is described.

Fluorine-18 labelled 3-(4,5-dihydrooxazol-2-yl)phenyl (5-fluoropentyl)carbamate, [^{18}F]**5**, was synthesized at high specific activity in a one-pot three step reaction using a commercial module with a radiochemical yield of 17–22% (from [^{18}F]fluoride). In vitro assay using rat brain homogenates showed that **5** inhibited FAAH in a time-dependent manner, with an IC_{50} value of 0.82 nM after a preincubation of 60 min. Ex vivo biodistribution studies and ex vivo autoradiography in rat brain demonstrated that [^{18}F]**5** had high brain penetration with standard uptake values of up to 4.6 and had a regional distribution which correlated with reported regional FAAH enzyme activity. Specificity of binding to FAAH with [^{18}F]**5** was high (>90%) as demonstrated by pharmacological challenges with potent and selective FAAH inhibitors and was irreversible as demonstrated by radioactivity measurements on homogenized brain tissue extracts.

We infer from these results that [^{18}F]**5** is a highly promising candidate radiotracer with which to image FAAH in human subjects using PET and clinical studies are proceeding.

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1. Introduction

The identification[1] and cloning[2] of the cannabinoid 1 (CB1) receptor took some time following the discovery of delta-9-tetrahydrocannabinol (THC) as the active psychotropic component of *Cannabis sativa*.[3] However, subsequent elucidations in the endocannabinoid system have been much more rapid[4–7] and the endocannabinoid system has emerged as an important target for basic neuroscience studies, as well as providing targets for therapeutic drugs.[5,7–12]

One of the problems associated with cannabinoids as therapeutic agents is their propensity to cause central psychotropic effects, and a proposed way around this has been the targeting of the enzymes regulating endocannabinoid levels. The enzyme fatty acid amide hydrolase (FAAH), which regulates the levels of the endogenous signaling molecule anandamide (AEA) may be useful in this respect.[13] Unlike 'classical' hydrophilic neurotransmitters lipophilic AEA is not stored in vesicles but rather is produced on demand and is rapidly degraded by FAAH to terminate signaling.[14,15] FAAH is found in many tissues, in particular the brain, liver, and kidney, and within the brain the activity varies across

Abbreviations: FAAH, fatty acid amide hydrolase; PET, positron emission tomography; CB1, cannabinoid 1 receptor; THC, delta-9-tetrahydrocannabinol; AEA, anandamide; SUV, standard uptake value; IC_{50}, concentration required to inhibit 50% of enzyme activity; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; HRMS, high resolution mass spectometry; DCM, dichloromethane; MeOH, methanol; $CDCl_3$, deuterated chloroform; EtOAc, ethyl acetate; DMSO, dimethylsulphoxide; CH_3CN, acetonitrile; EDTA, ethylenediaminetetraacetic acid.

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regions, with the highest activity being found in the hippocampus and cortex, and the lowest in the brain stem.[14,16] Selective inhibitors of FAAH have been actively pursued as a method to increase AEA levels and activate CB1 receptors in a focused manner, with the aim of providing therapeutic effects in a variety of disorders including pain, addiction, and obesity.[10,12,16–24] Such compounds do not produce the 'cannabis-like' behaviors seen with CB1 receptor agonists.[17,18]

In vivo imaging of the endocannabinoid system has also been pursued using positron emission tomography (PET) and a variety of radiotracers for the CB1 receptor have been successfully developed and translated into human PET studies.[19–23] For FAAH, a number of positron emitting radiotracers have been reported by us and others,[24–28] but only one has been validated for use in imaging FAAH in humans, namely [^{11}C]CURB.[29] While this radiotracer shows much promise, it is labelled with the short-lived radionuclide ^{11}C ($t_{1/2}$ = 20.4 min) and thus its use is confined to sites that have an on-site cyclotron for the production of ^{11}C. Fluorine-18 is the other commonly used radionuclide in PET and, with a half-live of 109.8 min, can be shipped and used at remote locations, thereby enabling multi-center trials.[30] We describe here the synthesis and radiosynthesis of a novel and potent FAAH inhibitor, 3-(4,5-dihydrooxazol-2-yl)-phenyl (5-fluoropentyl)carbamate, **5**, radiolabelled at high specific activity with ^{18}F. Evaluation in vitro and ex vivo in rats shows that [^{18}F]**5** is a potent FAAH inhibitor with excellent brain penetration, appropriate regional distribution, and high specific binding to FAAH.

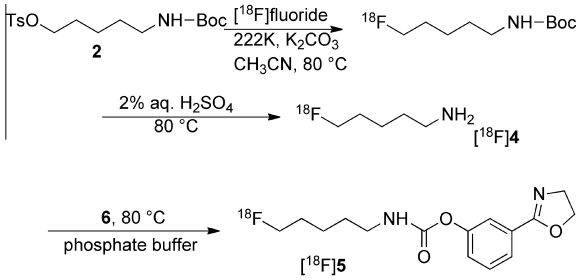
2. Results

2.1. Chemistry

Compound **5** was synthesised in four steps from 5-amino-1-pentanol (Scheme 1). Protection of the amino group of the amino alcohol was effected with *t*-Boc anhydride to form **1**, followed by fluorination with DAST, yielding **3**. Acid catalysed removal of the *t*-Boc group of **3** gave 5-fluoropentylamine **4** as the hydrochloride salt. Coupling of this fluoroamine with the *p*-nitrophenylcarbonate of 3-(4,5-dihydrooxazol-2-yl)phenol, **6**, provided **5** in an overall yield of 14% (four steps). The *t*-Boc protected tosylate of 5-amino-1-pentanol, **2**, which was required for radiolableling, was synthesised from **1** using *p*-toluenesulphonyl chloride in dichloromethane while the activated carbonate, **6**, was obtained by acylation of 3-(4,5-dihydrooxazol-2-yl)phenol with *p*-nitrophenylchloroformate in DMSO.

2.2. Radiochemistry

The radiosynthesis of [^{18}F]**5** is outlined in Scheme 2. Displacement of the tosyl group of **2** by [^{18}F]fluoride proceeded smoothly



Scheme 2. Radiosynthesis of [^{18}F]**5**.

at 80 °C in acetonitrile to give the *t*-Boc protected [^{18}F]-fluoropentylamine with incorporation yields of 70–80%. Removal of the *t*-Boc group was effected by addition of dilute sulfuric acid with 85–95% efficiency, yielding [^{18}F]**4**. After neutralising the acid with phosphate buffer, [^{18}F]**4** was coupled with carbonate **6** at 80 °C to generate [^{18}F]**5** in 70–80% radiochemical yield.

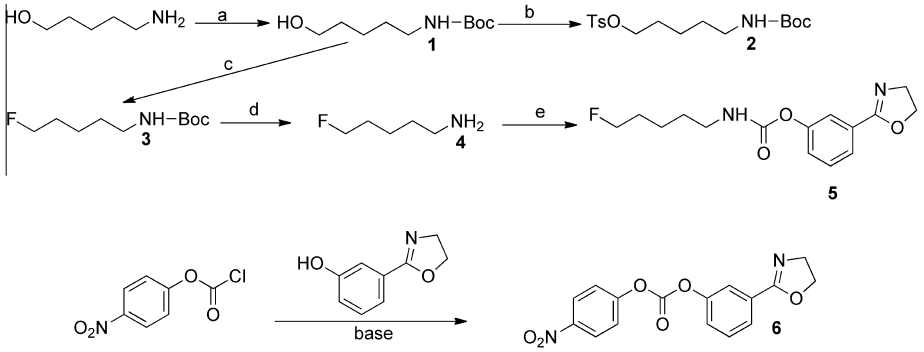
Reverse-phase HPLC purification (see SI, Fig. S1), followed by solid-phase formulation and sterile filtration, gave [^{18}F]**5** suitable for animal studies in an isolated uncorrected radiochemical yield (from [^{18}F]fluoride) of 17–22% (n = 7). The synthesis took 52–55 min from end-of-bombardment while the radiochemical purity was >97% with specific activities of 74–144 GBq/μmol at end-of-synthesis.

2.3. Inhibition of FAAH by compound 5

The ability of **5** to inhibit FAAH activity was measured using rat brain homogenates and [^{3}H]anandamide as substrate[50] (Fig. 1). Following a 60 min preincubation phase, the compound inhibited FAAH with a pI_{50} value of 9.09 ± 0.04, corresponding to an IC_{50} value of 0.82 nM (Fig. 1A). Its inhibitory potency was dependent upon the pre-incubation time used, a characteristic of irreversible enzyme inhibitors (Fig. 1B).

2.4. Biodistribution of [^{18}F]5 in rat brain

Upon tail vein injection of high specific activity [^{18}F]**5** (<1 nmol/kg) into conscious rats, brain uptake of radioactivity was rapid and high as measured by ex vivo dissection and counting of radioactivity in specific brain regions (Fig. 2). Based on previous studies, the calculated occupancy of the enzyme is less than 2% at these mass doses.{Wilson, 2011 #4101} At 2 min post-injection radioactivity in cortex (a FAAH rich region)[16] was 3.9 ± 0.70 SUV, which increased modestly to 4.55 ± 0.57 SUV at 40 min post-injection. The hypothalamus (a comparatively FAAH-poor region) had radioactivity levels of 1.74 ± 0.18 SUV and 1.59 ± 0.24 SUV at 2 and 40 min post-injection, respectively. To demonstrate that radiotracer uptake was mediated



Scheme 1. Synthesis of **5** and precursors required for radiosynthesis of [^{18}F]**5** and the activated carbonate, **6**. Reagents and conditions: (a) *t*-Boc anhydride, K_2CO_3, 74%; (b) TsCl, TEA, 82%; (c) DAST, DCM, 43%; (d) HCl in MeOH, 94%; (e) compound **6**, $KHCO_3$, 39%.

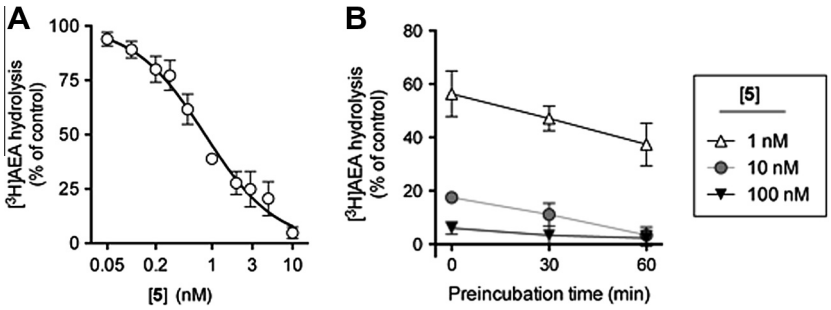


Figure 1. Inhibition of rat brain FAAH-catalysed [^{3}H]anandamide hydrolysis by compound **5**. Panel A shows a concentration–response curve using a preincubation time of 60 min, and Panel B shows the inhibition produced by three concentrations of **5** following different preincubation times. Given that the incubation time was 10 min, the inhibition seen at preincubation time zero is likely to have a time-dependent component. Data are means ± SEM, $n = 3$.

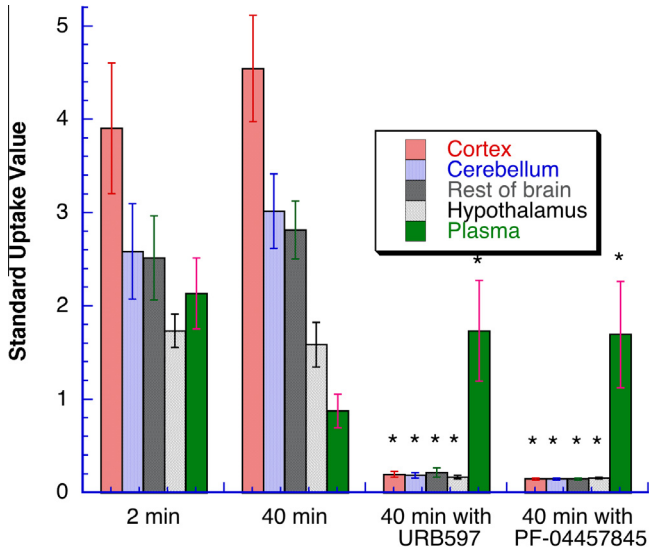


Figure 2. Biodistribution of [^{18}F]**5** in rat brain at 2 and 40 min post tail vein injection ($n = 4$/group, mean ± SD). The blocked groups received URB597 (2 mg/kg, ip) or PF-04457845 (0.5 mg/kg, ip) 30 min before radiotracer. $^*p$ <0.05.

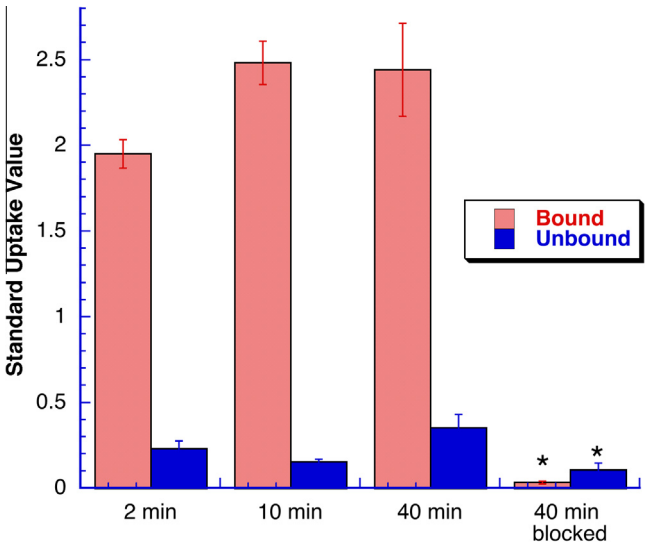


Figure 3. Amount of radioactivity irreversibly bound to rat brain tissue at 2, 10, and 40 min post tail vein injection of [^{18}F]**5** ($n = 4$–6/group, mean ± SD). The blocked group received URB597 (2 mg/kg, ip) 30 min before radiotracer. $^*p$ <0.05.

by FAAH binding, groups of rats were pretreated with either the prototypical carbamate FAAH inhibitor URB597 (2 mg/kg, ip) or the recently developed urea FAAH inhibitor, PF-04457845 (0.5 mg/kg, ip), 30 min prior to radiotracer administration.[31–35] Both inhibitors emphatically reduced uptake of radioactivity in all brain regions between 90% (hypothalamus) and 96% (cortex) with >90% reduction in uptake in the whole brain compared with controls (Fig. 2). Radioactivity levels in plasma were significantly elevated in pre-treated animals, an effect seen previously for FAAH radiotracers.[27,36]

2.5. Irreversible binding of [^{18}F]5 to rat brain parenchyma

Upon injection of [^{18}F]**5** to groups of conscious rats, whole brains were excised, homogenized, and exhaustively extracted with aqueous acetonitrile to differentiate irreversibly bound radiotracer from unbound.[37] Following centrifugation, radioactivities in the washed pellet (bound) and in the combined supernatant (unbound) were counted (Fig. 3). Even at 2 min post-injection, most (91%) of the radioactivity was irreversibly bound to brain parenchyma, an amount which did not change significantly at 10 min (94%) or 40 min (90%) post-injection, although the absolute amount of radioactivity bound did increase over this time period. Pretreatment of rats with URB597 (2 mg/kg, ip) 30 min prior to radiotracer injection reduced the amount of radioactivity irreversible bound by >98% compared with the control group and only 24% of total brain radioactivity was bound to tissue.

2.6. Metabolism of [^{18}F]5

Reverse-phase radio-HPLC analysis of rat plasma 10 min post-radiotracer injection showed that almost complete (>95%) metabolism had occurred, with the radioactivity associated with hydrophilic metabolites. However this analysis was compromised as we found the radiotracer to be unstable in rat plasma. Incubation of [^{18}F]**5** in vitro in rat plasma at ambient temperature resulted in >50% decomposition within 30 min (Fig. 4). In contrast, [^{18}F]**5** was stable in human plasma during the same time-period, with <5% decomposition, perhaps suggesting that decomposition in vitro in rat plasma was mediated by carboxylesterases as these enzymes are not present in human plasma.[35,38]

Radio-HPLC analysis of rat brain extracts, 10 min post-radiotracer injection showed that 50% of the extractable radioactivity was metabolized [^{18}F]**5** (data not shown). However it must be noted that extractable radioactivity represents <10% of the total radioactivity present in the brain (vide supra). In addition, as brains were non-perfused, the vasculature compartment of the rat brain (5%) represents a significant source of extractable radioactivity under these conditions.

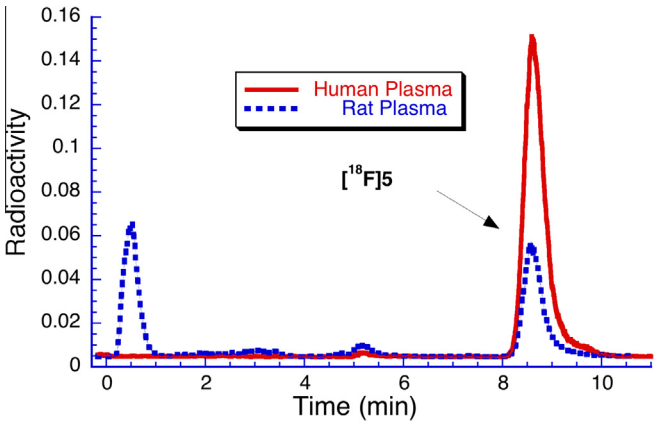


Figure 4. Radio-HPLC chromatograms of [^{18}F]**5** upon incubation in rat and human plasma for 30 min at ambient temperature.

2.7. Ex vivo autoradiography of [^{18}F]5 in rat brain

Figure 5 shows autoradiographic images of [^{18}F]**5** at the level of the forebrain (A) and cerebellum (B). As illustrated, considerable labelling was present in cortex, hippocampus, thalamic and amygdaloid nuclei (A) as well as cerebellar cortex (B). Signal was also clearly seen in the ventricular space, which is consistent with previously reports of expression of FAAH in the choroid plexus.[39–41] Pretreatment with 0.5 mg/kg of PF-04457845 (C and D) completely abolished binding in all brain regions, indicating exceptional specificity of the radiotracer for its target sites ex vivo.

3. Discussion

Our target compound, 3-(4,5-dihydrooxazol-2-yl)phenyl (5-fluoropentyl)carbamate, **5**, (log *D*, pH 7.4 of 2.33) was chosen on the basis of previous preclinical studies with ^{11}C-labelled FAAH inhibitors which showed that *N*-alkyl-3-(4,5-dihydrooxazol-2-yl)phenyl carbamates possess excellent properties as FAAH imaging radiotracers, including high brain uptake, selectivity, and fast kinetics.[28] In vitro enzyme assays showed that **5** was also a potent and irreversible inhibitor of FAAH with an IC_{50} of 0.8 nM, akin to its non-fluorinated analogues.[28] While one-step radiolabelling is always desirable, especially when dealing with short-lived radioisotopes, direct introduction of ^{18}F into **5** using arylsulphonate esters as precursors proved unfruitful. Under the basic conditions required for

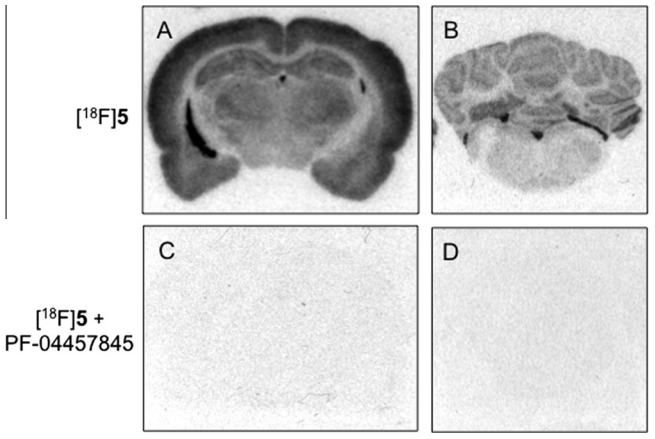


Figure 5. Ex vivo autoradiography of [^{18}F]**5** in rat brain; control (A and B) and blocked (C and D). See Figure S2 for more slices.

^{18}F-nucleophilic substitution reactions, such aryl carbamates undergo a facile and rapid E2 elimination reaction which precluded introduction of [^{18}F]fluoride directly.[42] However, judicious choice of reaction conditions that efficiently generated a synthetically versatile [^{18}F]fluoroamine, [^{18}F]**4**, allowed [^{18}F]**5** to be prepared in an efficient one-pot three-step reaction (Scheme 1), which was easily automated using a commercial apparatus.

The suitability of [^{18}F]**5** as a radiotracer for imaging FAAH by PET was demonstrated in several ways:

(1) Ex vivo biodistribution studies showed that brain penetration of [^{18}F]**5** was high and mediated by FAAH as pre-treatment with established and selective FAAH inhibitors URB597 or PF-04457845 blocked >90% of brain uptake (Fig. 2).

(2) The brain regional uptake of radioactivity 40 min post administration of [^{18}F]**5** correlated well with the reported regional FAAH enzyme activity in rat brain. A similar correlation was found with the clinically used ^{11}C-radiotracer [^{11}C]CURB and related analogs,[26] as well as the closely related congener of **5**, [^{11}C]**8** reported elsewhere (Fig. 6).[28] This is direct evidence that regional uptake of [^{18}F]**5** is sensitive to FAAH activity.

(3) FAAH is inactivated by *N*-alkyl-*O*-arylcarbamates by carbamoylation of the Ser_{241} amino acid, resulting in ejection of the *O*-aryl residue.[43,44] Thus it was anticipated that introduction of the radiolabel on the *N*-alkyl chain of **5** would result in the irreversible tagging of FAAH with ^{18}F upon binding. To demonstrate this, excised rat brain was homogenized and repeatedly extracted with solvent to measure the amount of radiolabel irreversibly bound to rat brain parenchyma following radiotracer injection (Fig. 3).[37] Less than 10% of total radioactivity could be extracted, demonstrating the irreversible nature of the binding. This irreversible binding was mediated by FAAH as demonstrated by its almost complete abolishment by pre-treatment of animals with URB597. Scheme 3 depicts the putative mechanism of action of [^{18}F]**5**.

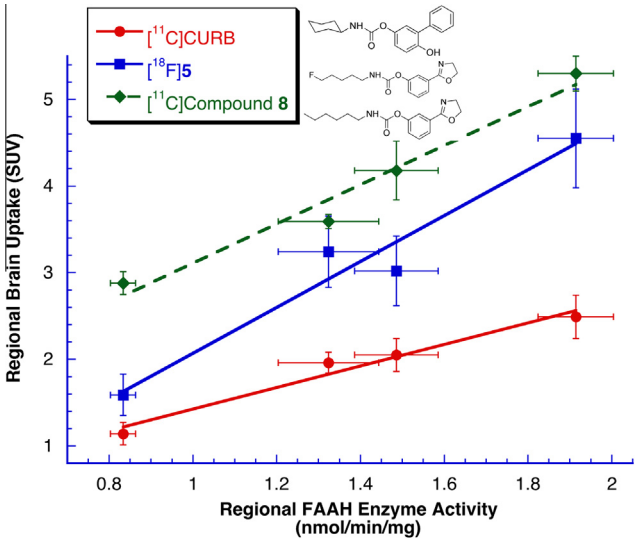
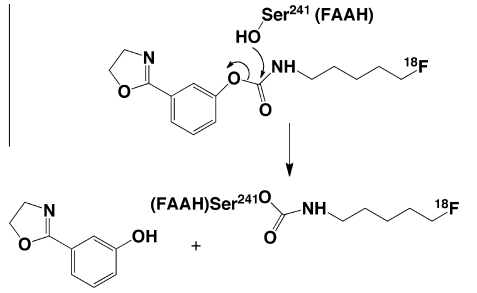


Figure 6. Correlation between regional brain uptake of FAAH radiotracers and regional FAAH enzyme activity in rat brain. Data taken from this work ([^{18}F]**5** uptake); Wilson et al., ([^{11}C]CURB and compound [^{11}C]**8** uptake);[26,28] and Thomas et al., (FAAH enzyme activity).[16] Error bars represent standard deviation from the mean.



Scheme 3. Putative mechanism of irreversible binding of [^{18}F]**5** to FAAH. Carbamoylation of the serine241 residue results in FAAH tagged with the [^{18}F]fluoropentylcarbamoyl group.

(4) The distribution of radioactivity after injection of [^{18}F]**5** as depicted by ex vivo autoradiography studies are in close agreement with previous autoradiography, immunohistochemical, and immunoreactivity studies which measured the patterns of FAAH activity, mRNA, and expression in rat brain, including high levels in the ventricles (Fig. 5).[16,39,41,45] Specificity of uptake was again demonstrated by the complete disappearance of binding following pretreatment with a selective FAAH inhibitor.

4. Conclusions

We have identified and characterized, in vitro and ex vivo in rats, a novel ^{18}F-labelled radiotracer for PET imaging of FAAH. [^{18}F]**5** shows high brain uptake upon intravenous administration with binding which is sensitive to FAAH regional activity. As demonstrated with dissection studies and ex vivo autoradiography, [^{18}F]**5** binds to FAAH with a high degree of selectivity and specificity. These properties suggest that the radiotracer is eminently suitable for neuroimaging of FAAH in the living human brain using PET. Clinical studies with [^{18}F]**5** are being pursued in our laboratory.

5. Experimental methods

5.1. Chemistry

A Scanditronix MC 17 cyclotron was used for radionuclide production. Purifications and analyses of radioactive mixtures were performed by high performance liquid chromatography (HPLC) with an in-line UV (254 nm) detector in series with a NaI crystal radioactivity detector (purifications) or a Bioscan Flowcount coincidence radioactivity detector (analyses). Isolated radiochemical yields were determined with a dose-calibrator (Capintec CRC-712M). Unless stated otherwise, all radioactivity measurements were normalised for radioactive decay. Proton and carbon-13 NMR spectra were recorded at 25 °C on a Varian Mercury 400 MHz spectrometer. Electrospray ionization mass spectrometry was conducted with MDS Sciex QStar mass spectrometer to obtain the HRMS. All tested compounds had a purity of >95% as determined by reverse-phase HPLC. All animal experiments were carried out under humane conditions, with approval from the Animal Care Committee at the Centre for Addiction and Mental Health, and in accordance with the guidelines set forth by the Canadian Council on Animal Care. Rats (male, Sprague–Dawley, 300–350 g) were kept on a reversed 12-h light/12-h dark cycle and allowed food and water ad libitum.

5.2. *tert*-Butyl (5-hydroxypentyl)carbamate (1)

A mixture of 5-amino-1-pentanol (3.0 g, 29.1 mmol), *t*-Boc anhydride (6.1 g, 34.9 mmol), and K_2CO_3 (4 g) in 50/50 water/methanol (25 mL) was stirred at ambient temperature for 2 h. The reaction mixture was quenched with water (25 mL) and extracted with EtOAc (2 × 25 mL). Combined extracts were washed with water and brine, dried ($MgSO_4$), and filtered. Solvent removal gave the product as a viscous colourless oil (4.4 g, 74%). ^{1}H NMR ($CDCl_3$): δ ppm 1.3–1.63 (m, 15H) 3.07–3.18 (m, 2H) 3.65 (t, J = 6.44 Hz, 2H) 4.55 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ ppm 22.89, 28.38, 29.80, 32.20, 40.39, 62.53. 79.07, 156.08. HRMS (ESI$^+$) m/z calcd for $[M+H]^+$ $C_{10}H_{22}NO_3$ 204.15997, found 204.16070.

5.3. 5-((*tert*-Butoxycarbonyl)amino)pentyl 4-methylbenzenesulfonate (2)

A mixture of *tert*-butyl (5-hydroxypentyl)carbamate (1.5 g, 7.4 mmol), *p*-toluenesulfonyl chloride (1.5 g, 7.9 mmol), triethylamine (100 μL, 0.72 mmol), and potassium carbonate (2 g, 14.5 mmol) in dichloromethane (10 mL) was stirred at ambient temperature for 3 days. Water (100 mL) was added and the mixture extracted with ethyl acetate (2 × 50 mL). The combined organic extracts were washed with brine, dried (Na_2SO_4), filtered and solvent removed to leave a light brown oil (2.41 g). Flash chromatography (hexane/EtOAc, 30:70) gave a colourless oil from heart fractions which crystallised upon trituration with pentane to give the product as a white solid (0.97 g, 37%). A further 1.2 g (45%) was isolated from other fractions. Mp 47–48 °C. ^{1}H NMR ($CDCl_3$): δ ppm 1.30–1.38 (m, 2H) 1.40–1.48 (m, 11H) 1.61–1.71 (m, 2H) 2.45 (s, 3H) 3.06 (q, J = 6.56 Hz, 2H) 4.02 (t, J = 6.33 Hz, 2H) 4.50 (br s, 1H) 7.35 (m, J = 7.99 Hz, 2H) 7.79 (m, 2H). ^{13}C NMR ($CDCl_3$): δ ppm 21.85, 22.88, 28.62, 29.65, 40.44, 70.55, 79.36, 128.10, 130.05, 133.35, 144.94, 156.14. HRMS (ESI$^+$) m/z calcd for $[M+H]^+$ $C_{17}H_{28}NO_5S$ 358.1683, found 358.1679.

5.4. *tert*-Butyl (5-fluoropentyl)carbamate (3)

To a stirred solution of DAST (1.87 ml, 19 mmol) in DCM (5 mL) at −70 °C was added a solution of *tert*-butyl (5-hydroxypentyl) carbamate (3 g, 14 mmol) in DCM (6 mL) over 30 min. The mixture was stirred for 12 h at room temperature, quenched with saturated $NaHCO_3$ (25 mL) and the organic layer separated. The aqueous phase was extracted with DCM (10 mL) and the combined organic extracts dried ($MgSO_4$), filtered, and volatiles evaporated. The product was obtained as a colourless oil upon purification by silica column chromatography with DCM/hexane 1/1 as mobile phase. (1.3 g, 43%) ^{1}H NMR ($CDCl_3$): δ ppm 1.39–1.57 (m, 13H) 1.62–1.78 (m, 2H) 3.12 (q, J = 6.24 Hz, 2H), 4.43 (dt, J = 47.9 Hz, 6.05 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ ppm 22.47 (d, J = 5.37 Hz) 28.37, 29.67, 30.00 (d, J = 19.94 Hz), 40.35, 79.03, 83.87 (d, J = 164.09 Hz,) 155.96. ^{19}F NMR ($CDCl_3$) δ ppm) 47.59 (tt, J = 47.30, 25.94 Hz). HRMS (ESI$^+$) m/z calcd for $[M+H]^+$ $C_{10}H_{21}FNO_2$ 206.15563, found 206.15582.

5.5. 5-Fluoropentylamine hydrochloride (4)

A solution of HCl in MeOH (3 M, 6 mL) was slowly added to a solution of *tert*-butyl (5-fluoropentyl)carbamate (0.100 g, 0.49 mmol) in $CHCl_3$ (5 mL) at room temperature. After 12 h, volatiles were evaporated to give 5-fluoropentylamine hydrochloride (0.065 g, 94%) as a light brown hygroscopic solid. ^{1}H NMR (MeOH-d_4): δ ppm 1.43–1.58 (m, 2H), 1.65–1.84 (m, 4H), 2.95 (t, J = 7.61 Hz, 2H), 4.45 (dt, J = 6.05, 47 .4 Hz, 2H) 4.51. ^{13}C NMR (MeOH-d_4): δ ppm 22.02 (d, J = 5.37 Hz), 26.75, 29.56 (d, J = 19.94 Hz), 39.27, 83.18 (d, J = 164.09 Hz) ^{19}F NMR (MeOH-d_4) δ ppm) 45.69 (m). HRMS (ESI$^+$) m/z calcd for $[M+H]^+$ $C_5H_{13}F$ 106.10320, found 106.10296.

5.6. 3-(4,5-Dihydrooxazol-2-yl)phenyl (5-fluoropentyl) carbamate (5)

A solution of 5-fluoropentylamine hydrochloride (95 mg, 0.67 mmol) in CH_3CN/H_2O (4:1, 5 mL) was added to a stirred mix-

ture of 3-(4,5-dihydrooxazol-2-yl)phenyl (4-nitrophenyl) carbonate (170 mg, 0.52 mmol) and $KHCO_3$ (200 mg, 2.0 mmol) in CH_3CN (5 mL). The mixture was stirred at room temperature for 3 h then DCM (10 mL) and satd aqueous $NaHCO_3$ (50 mL) were added. The aqueous layer was extracted with DCM and the combined organic layers filtered and solvent removed to leave a light brown oil (143 mg). Flash chromatography (hexane/EtOAc; 50:50) on silica gel gave a colourless oil which upon trituration with 20 mL hexane gave the desired product as a white solid (72 mg, 47% yield); mp 74.5–77 °C. ^{1}H NMR ($CDCl_3$): δ ppm 1.46–1.54 (m, 2H), 1.60–1.81 (m, 4H), 3.28 (q, J = 6.63 Hz, 2H), 4.05 (t, J = 9.56 Hz, 2H), 4.37–4.46 (m, 3H), 4.52 (t, J = 5.85 Hz, 1H), 5.19 (br s, 1H), 7.23–7.29 (m, 1H), 7.39 (t, J = 8.00 Hz, 1H), 7.70 (s, 1H), 7.78 (d, J = 7.80 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ ppm 22.30 (d, J = 5.37 Hz), 29.27, 29.81 (d, J = 19.94 Hz), 40.89, 54.74, 67.53, 83.67 (d, J = 164.86 Hz), 121.30, 124.42, 124.82, 128.84, 129.05, 150.77, 154.15, 163.79. ^{19}F NMR ($CDCl_3$): d ppm 47.39 (m). HRMS (ESI$^+$) m/z calcd for $[M+H]^+$ $C_{15}H_{20}FN_2O_3$ 295.14580, found 295.14450.

5.7. 3-(4,5-Dihydrooxazol-2-yl)phenyl (4-nitrophenyl) carbonate (6)

A solution of *p*-nitrophenylchloroformate (120 mg, 0.6 mmol) in CH_3CN (2 mL) was added to a stirred solution of 3-(4,5-dihydrooxazol-2-yl)phenol[46] (100 mg, 0.6 mmol) and DIPEA (110 μL, 0.63 mmol)) in CH_3CN (2 mL) and DMSO (0.8 mL). The mixture was stirred at ambient temperature for 20 min then quenched with 5% citric acid (17 mL). The resultant precipitate was collected by vacuum filtration and washed with copious amounts of water. Drying in vacuo gave the product as a pale yellow powder which was purified by flash chromatography (hexane/EtOAc, 80:20 to 30:70) yielding a white solid (77 mg, 39%); mp 150–152 °C. ^{1}H NMR ($CDCl_3$): δ ppm 4.10 (t, J = 9.65 Hz, 2H), 4.47 (t, J = 9.65 Hz, 2H), 7.38–7.43 (m, 1H), 7.47–7.53 (m, 3H), 7.88–7.93 (m, 2H), 8.30–8.36 (m, 2H). ^{13}C NMR ($CDCl_3$): δ ppm 55.02, 67.90, 120.73, 121.74, 123.52, 125.43, 126.44, 129.69, 129.78, 145.69, 150.52, 150.87, 155.20, 163.45. HRMS (ESI$^+$) m/z calcd for $[M+H]^+$ $C_{16}H_{13}N_2O_6$ 329.0768, found 329.0763.

5.8. Radiosynthesis of [^{18}F]5

Procedures were initially carried out by hand using <1 MBq of [^{18}F]fluoride to optimize reaction conditions. Subsequently the radiosynthesis was carried out using a radiosynthesis module (for details see[47]) using 14–20 GBq. [^{18}F]Fluoride was produced by the ^{18}O(p, n)^{18}F nuclear reaction using >95% enriched [^{18}O]H_2O. The [^{18}F]fluoride was trapped on a Chromafix ion-exchange 30-PS-HCO_3 resin, and eluted into a glass reactor with a solution consisting of 14.4 mg of 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (Kryptofix 222,) and 3 mg of K_2CO_3 in 1 mL of 4% (v/v) water in acetonitrile.[48] The solution was dried by a combination of heat (90 °C), vacuum, and N_2 flow (350 mL/min) for 5 min. After cooling the reactor to 60 °C, a solution of **2**, (5 mg. 14 μmol) in CH_3CN (0.5 mL) was added with stirring and heated to 80 °C for 7 min. Then 2% aq H_2SO_4 (0.5 mL) was added, heating and stirring continued for a further 7 min, and the solution neutralized with phosphate buffer (0.5 mL, prepared by dissolving K_2HPO_4 (34.84 g) and KOH (2.81 g) in water (250 mL)). A solution of **6** (7 mg, 21.3 μmol) in 70% aq. CH_3CN (1 mL) was added, with heating at 80 °C and stirring continued for a further 7 min. The mixture was cooled to <45 °C, quenched with water (2 mL), and purified by HPLC using a Phenomenex Gemini C18 5 μm, 250 × 10 mm column with 35:65 CH_3CN/H_2O + 0.1 N ammonium formate as eluent at 7 mL/min. The fraction containing [^{18}F]**5** was collected, diluted with 25 mL of water, and trapped on a pre-conditioned reverse-phase cartridge (Waters Sep-Pak tC18+). After washing the cartridge with water (5 mL) to remove salts and residual acetonitrile, it was eluted with ethanol (1 mL), chased with isotonic citrate buffer (10 mL, pH 6), prepared by dissolving trisodium citrate dehydrate (0.588 g) and disodium hydrogen citrate 1.5 hydrate (0.263 g) in water (100 mL), and passed through a sterile 0.2 μm filter into a sterile, pyrogen-free bottle. Co-injection of the radioactive product with an authentic standard of **5** under several reverse-phase conditions, varying solvents (methanol, acetonitrile, THF) pH, (7.0, 5.0, 4.0) with several analytical HPLC columns (Waters, Grace, Phenomenex, ThermoScientific, C18 and C8) established the identity of the radiotracer. Under all conditions [^{18}F]**5** co-chromatographed with authentic **5**. The formulated radiotracer displayed <5% radiolysis 120 min post formulation and proved to be sterile and pyrogen-free, suitable for animal studies.

5.9. Log *P* measurements

The partition coefficients of [^{18}F]**5**, between 1-octanol and 0.02 M phosphate buffer at pH 7.4, was determined to be 2.33 ± 0.01 by a previously described method, replicated eight times.[49]

5.10. FAAH assays in vitro

FAAH activity was assayed in rat brain homogenates as described previously.[50] Briefly, **5** (in ethanol) was added to homogenates of frozen brains (minus cerebellum; 0.5 μg per assay) from adult rats (Wistar or Sprague–Dawley) in 50 mM Tris–HCl buffer, pH 7.4 containing 1 mM EDTA and 3 mM $MgCl_2$ and preincubated at 37 °C for the times shown in Figure 1. [^{3}H]Anandamide, labelled in the ethanolamine part of the molecule (American Radiolabeled Chemicals, Inc., St. Louis, MO) in 10 mM Tris–HCl, 1 mM EDTA, pH 7.4, containing 1% w/v fatty acid-free bovine serum albumin was then added to give a final substrate concentration of 0.5 μM and assay volume of 200 μl. The assay tubes were then incubated for 10 min at 37 °C after which reactions were stopped by placing the tubes on ice and adding activated charcoal (80 + 320 μl 0.5 M HCl). After mixing and phase separation, aliquots of the supernatants were analysed for tritium content by liquid scintillation spectroscopy with quench correction. Blanks contained assay buffer in place of enzyme homogenate.

5.11. Biodistribution studies in rats

Rats, in a restraining box, received 3–4 MBq of high specific activity [^{18}F]**5** (0.2–1 nmol/kg) in 0.3 mL of 10% ethanol in citrate buffer (pH 6) via the tail vein which had been vasodilated in a warm water bath. Groups of rats (n = 4–6) were sacrificed by decapitation at various time intervals after radiotracer administration, the brain surgically removed from the skull and stored on ice. Brain regions were excised, blotted, and weighed while blood was collected (from the trunk). Radioactivity in tissues was assayed in an automated gamma counter, back corrected to time of injection, using diluted aliquots of the initial injected dose as standards. Values are reported as standard uptake values (SUVs, mean ± standard deviation) defined as % injected dose/g of tissue divided by rat weight in kg. Treated groups were compared to those of the vehicle-treated group by Student's t test. Statistical comparisons were considered significant when p <0.05. For pharmacological challenge studies in rats, groups of rats (n = 4) were pre-treated with solutions of the challenge drug (2 mL/kg in 5% Tween 80 in saline ip) or vehicle 40 min prior to radiotracer injection. Animals were sacrificed 40 min post radiotracer administration and radioactivity in tissues measured as describe above.

5.12. Irreversible binding of [^{18}F]5 to FAAH and metabolite studies

Following tail-vein injection of [^{18}F]**5** groups of 4–6 rats were sacrificed at various timepoints and the whole brain was surgically

removed from the skull, washed in saline, and kept on ice. Rats in one group were pretreated with URB597 (2 mg/kg, 2 mL/kg in saline with 5% Tween80®, ip) 1 h prior to radiotracer injection to measure specific binding. Brains were then homogenized (Polytron, setting 7) in 5 mL of cold 80% acetonitrile/20% aqueous hydrochloric acid (0.01%) and centrifuged (17,000 rpm, 10 min). Following careful decantation of the supernatants, the pellets were resuspended in extraction solvent (5 mL) and centrifuged again. After repeating the extraction procedure once more, aliquots from the combined supernatants from each rat was removed, weighed and counted for radioactivity. Pellets were also counted for radioactivity. For metabolism studies blood was collected from the trunk in a heparinised tube and centrifuged to separate the plasma, which was treated with 10% by volume of acetic acid. Plasma and extracts of homogenized brain were directly analysed by HPLC analysis using minor modifications of the method described by Hilton.[26,51]

5.13. Autoradiography experiments

Male Sprague–Dawley rats with body weights ranging from 310 to 330 g were injected in the tail vein with [^{18}F]**5** (45–55 MBq, 0.7–0.8 nmol/kg). Forty minutes prior to the radiotracer injection, some of the animals were pretreated with vehicle (5% Tween80® in saline) or PF-04457845 (0.5 mg/kg, ip). All rats were sacrificed 40 min after the radiotracer injection. Brains were rapidly removed, frozen over dry ice, sliced into coronal 20-μm sections at −20 °C, and thaw-mounted onto Fisher Superfrost slides. Slides were quickly dried on a slide warmer and then exposed to Kodak Biomax film for 6 days. The average time between sacrifice and beginning of film exposure was 70 min.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.04.077. These data include MOL files and InChiKeys of the most important compounds described in this article.

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