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► To cite this version:

Benjamin Vidal, Iuliia Karpenko, François Liger, Sylvain Fieux, Caroline Bouillot, et al.. [11C]PF-3274167 as a PET radiotracer of oxytocin receptors: Radiosynthesis and evaluation in rat brain. Nuclear Medicine and Biology, 2017, 55, pp.1-6. 10.1016/j.nucmedbio.2017.07.008 . hal-01689748

HAL Id: hal-01689748

<https://hal.science/hal-01689748v1>

Submitted on 23 Jan 2025

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[¹¹C]PF-3274167 as a PET radiotracer of oxytocin receptors: Radiosynthesis and evaluation in rat brain

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ARTICLE INFO

Article history:

Received 21 April 2017

Received in revised form 24 July 2017

Accepted 29 July 2017

Keywords:

Oxytocin receptor

Positron emission tomography

Brain

Rat

[¹¹C]PF-3274167

ABSTRACT

Introduction: Oxytocin plays a major role in the regulation of social interactions in mammals by interacting with the oxytocin receptor (OTR) expressed in the brain. Furthermore, the oxytocin system appears as a possible therapeutic target in autism spectrum disorders and other psychiatric troubles, justifying current pharmacological researches. Since no specific PET radioligand is currently available to image OTR in the brain, the aim of this study was to radiolabel the specific OTR antagonist PF-3274167 and to evaluate [¹¹C]PF-3274167 as a potential PET tracer for OTR in rat brains.

Methods: [¹¹C]PF-3274167 was prepared via the *O*-methylation of its desmethyl precursor with [¹¹C]methyl iodide. The lipophilicity of the radioactive compound was evaluated by measuring the *n*-octanol-buffer partition coefficient (logD). Autoradiography experiments were performed on rat brain tissue to evaluate the *in vitro* distribution of the [¹¹C]PF-3274167. MicroPET experiments were conducted with and without pre-injection of ciclosporin in order to evaluate the influence of the P-glycoprotein (P-gp) on the brain uptake.

Results: [¹¹C]PF-3274167 was synthesized with high radiochemical and chemical purities (>95%) and good specific activity. The measured logD was 1.93. *In vitro*, [¹¹C]PF-3274167 did not show any evidence of specific binding to OTR. PET imaging showed that [¹¹C]PF-3274167 uptake in rat brain was very low in basal conditions but increased significantly after the administration of ciclosporin, suggesting that it is a substrate of the P-gp. In the ciclosporin-pre-injected rat, however, [¹¹C]PF-3274167 distribution did not match with the known distribution of OTR in rats.

Conclusion: [¹¹C]PF-3274167 is not a suitable tracer for imaging of OTR in rat brain, probably because of a too low affinity for this receptor in addition to a poor brain penetration.

1. Introduction

Recent research on the neuropeptide oxytocin has led to increasing evidence that it plays a key role as a neuromodulator in the central nervous system. Numerous studies have shown that oxytocin is involved in the regulation of a wide range of social behaviors via the stimulation of central receptors in mammals, from rodents to humans [1–5]. Furthermore, the oxytocin system appears as an attractive therapeutic target to improve social interactions in autism spectrum disorders and other

psychiatric troubles [6,7]. However, current knowledge of this system is limited, notably by the absence of a specific positron emission tomography (PET) radioligand, needed to visualize and quantify oxytocin receptors (OTR) *in vivo* in the brain. Although several candidates have been synthesized and evaluated in rodent and non-human primate, none of them was selected as a suitable radiotracer mainly because of difficulties to cross the blood–brain barrier [8–12]. The recently evaluated antagonist [¹⁸F]ALS-I-41 was the first attempt to display some visible binding in regions described as rich in oxytocin receptors in macaque [13]. However, brain uptake was still very low, justifying the need to evaluate other ligands as radiotracers for PET imaging of OTR.

In this context, we focused our interest on the triazole derivative PF-3274167, which was previously reported as a potent oxytocin receptor antagonist (K_i = 9.5 nM) with a high selectivity for the oxytocin versus

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the vasopressin receptors [14]. Data obtained from *in vitro* assays with MDCK cells also predicted a good CNS penetration of the compound [15].

We report here the radiosynthesis of [^{11}C]PF-3274167 and its pre-clinical evaluation in rats as a potential PET tracer for the brain oxytocin receptor. The radiolabeling was performed on the *O*-methyl group of the compound via a desmethyl precursor. The radiopharmacological evaluations included lipophilicity determination, autoradiography experiments on rat brain slices and *in vivo* microPET scans in isoflurane-anesthetized rats.

2. Materials and methods

2.1. Precursor synthesis

The desmethyl precursor **1** (Fig. 1A), was obtained according to the previously described protocol [16].

2.2. PET radiosynthesis and radiopharmaceutical quality controls

Using a $^{14}\text{N}[\text{p},\alpha]^{11}\text{C}$ nuclear reaction, no-carrier-added [^{11}C]carbon dioxide was produced in a IBA Cyclone-18/9 cyclotron by irradiation of a pressurized target (250 psi, 80 mL) containing a mixture of N_2/O_2 (99.5/0.5) with an 18 MeV proton beam. The radiomethylation was performed on an automated synthesis module (Scansys, Vaerloese, Denmark). The [^{11}C]iodomethane has been produced by a gas phase method [17] (gas flow during trapping: 25 mL/min) and was bubbling in a solution of 1.1 mg of free-alcohol precursor **1** and 2 mg of NaH in dry DMF (300 μL). The mixture was heated at 90 $^\circ\text{C}$ for 5 min. After dilution with 2.5 mL of HPLC eluent, the resulting mixture was purified

by preparative HPLC (C18 Sunfire Waters, 5 μm , 10 mm \times 250 mm), eluting with H_3PO_4 0.1M/ CH_3CN , (60/40; pH = 1.9), at 3 mL \cdot min $^{-1}$ ($\lambda = 254$ nm). For biological use, the radiotracer was formulated via SPE techniques [18]. The product was diluted in 40 mL of sterile water and loaded on a SEP-Pak Light C18 cartridge (Waters, Milford, MA, U.S.). The loaded cartridge was rinsed with water and eluted with 1 mL of ethanol and 2 mL of water, and the final product was diluted with isotonic saline and sterilized by filtration (sterile filter Millex-GS, 0.22 μm). The radiochemical purity and specific activity of [^{11}C]PF-3274167 were assayed by analytical HPLC (MachereyNagel EC 250/4.6 Nucleodur 100-5-C18ec C18 column; mobile phase $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{TFA}$, 55/45/0.1 %; flow rate, 0.9 mL \cdot min $^{-1}$). The identity of [^{11}C]PF-3274167 was confirmed by co-injection with an authentic nonradioactive sample (synthesized in house in LIT, Strasbourg).

2.3. Animals

Adult male Sprague–Dawley rats (Charles River Laboratories; 300 \pm 50 g) were used. All experiments were performed in accordance with European guidelines for care of laboratory animals (2010/63/EU) and were approved by the ethics animal committee of the Université de Lyon.

2.4. Partition coefficient measurement

Lipophilicity at physiological pH (logD) was estimated by partitioning between *n*-octanol and buffer (50 mmol/L of Tris-HCl, pH adjusted to 7.4). LogD was determined as the \log_{10} of the concentration of radiotracer in *n*-octanol over the concentration in buffer (measured

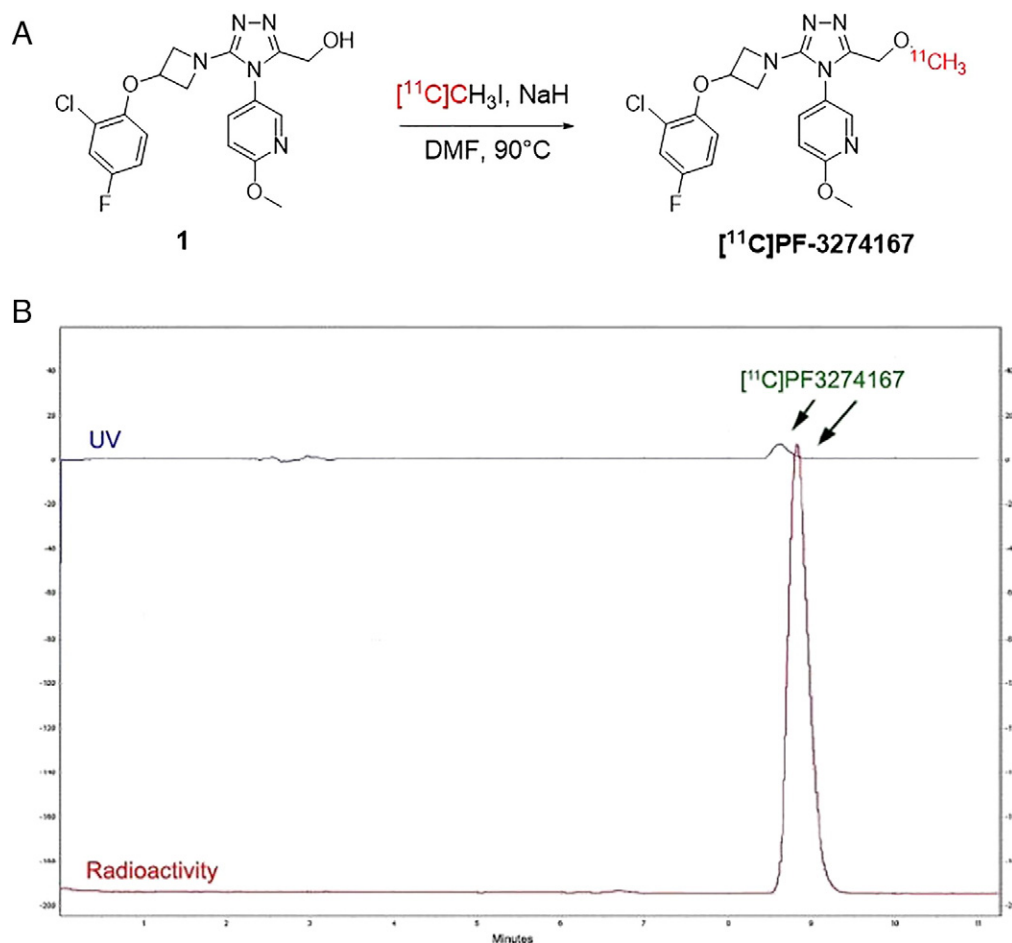


Fig. 1. A. Radiosynthesis of [^{11}C]PF-3274167. B. Analytical HPLC chromatogram of [^{11}C]PF-3274167 (Ultraviolet absorbance at 254 nm in blue and radioactivity in red).

by γ counting of samples of each phase). This value of logD was confirmed by calculation with the ACD/Labs software (7.09 version, Advanced Chemistry Development Inc.).

2.5. Autoradiography studies

After a short inhalation of isoflurane, rats were decapitated and brains were removed and immediately frozen in 2-methylbutane cooled with dry ice ($-29\text{ }^{\circ}\text{C}$). Coronal sections ($30\text{ }\mu\text{m}$ thick) were cut using a $-20\text{ }^{\circ}\text{C}$ cryostat, thaw-mounted on glass slides, and allowed to air dry before storage at $-80\text{ }^{\circ}\text{C}$ until used. The day of radiosynthesis, the slides were incubated for 20 min in Tris phosphate-buffered saline buffer (TBS), pH adjusted to 7.4, containing 37 kBq/mL of [^{11}C]PF-3274167. For competition experiments, the slides were placed in the same buffer supplemented with increasing concentrations of unlabeled PF-3274167 or the OTR partial agonist carbetocin (1, 10 and 100 nM). After incubation, the slides were dipped two times in TBS buffer (either alone or half-mixed with ethanol at 96°) for 90 s, then immediately dipped in distilled cold water ($4\text{ }^{\circ}\text{C}$) and dried and juxtaposed to a phosphor imaging plate for 90 min (BAS-1800 II; Fujifilm). Regions of interest (ROIs) were drawn manually using Multigauge software (Fujifilm) according to a rat brain atlas [19]. The results were expressed in optical densities (PSL/ mm^2) or in percentage of control.

2.6. MicroPET studies

Rats were anesthetized with 4%–5% isoflurane for 5 min (induction phase), and a catheter was placed into their caudal vein. Anesthesia was maintained to 2% isoflurane during the acquisition on the PET/CT camera (Inveon, Siemens), with continuous monitoring of the respiratory rate using a pressure sensor. The acquisition started with a CT image acquisition for 10 min, followed by the intravenous injection of [^{11}C]PF-3274167 ($36 \pm 11\text{ kBq/g}$). The total duration of the scan was 60 min. The images were reconstructed in three dimensions in a series of 24 sequential frames of increasing duration from 10 s to 5 min. ROIs were automatically delineated using a multi-atlas dataset [20] after manual positioning of the PET images on an anatomical MRI template using the software Inveon Research Workplace (IRW, Siemens). The time–activity curves were expressed in Bq/cm^3 normalized to the injected dose corrected for weight, to obtain standardized uptake values (SUVs). To study the influence of P-glycoprotein (P-gp) on the brain uptake of the tracer, one rat received an intravenous injection of ciclosporin (Sandimmun®, Novartis) at 50 mg/kg 30 min before the tracer injection. The same rat was used for a control acquisition, with an intravenous injection of saline 30 min before acquisition. One rat pre-treated with ciclosporin also received an intraperitoneal (i.p) injection of WAY-267,464 (Tocris), a partial oxytocin receptor agonist, at 30 mg/kg 20 min before tracer injection.

3. Results

3.1. Radiosynthesis and quality control

Radiosynthesis of [^{11}C]PF-3274167 from the precursor **1** (Fig. 1A) was performed with a radiochemical yield of 55% to 70% corrected for decay (based on ^{11}C -iodomethane) and an overall time of 50 min. No radioactive by-products were observed, and the quality control confirmed the separation of the [^{11}C]PF-3274167 from its precursor (Fig. 1B). Radiochemical and chemical purity was at least 95% (no other compounds could be detected) and specific activity ranged between 55 and $111\text{ GBq }\mu\text{mol}^{-1}$, corrected at EOS.

3.2. Partition coefficient measurement

The n-octanol-buffer partition coefficient (logD), reflecting lipophilicity, was 1.93 ± 0.01 (experiments performed in duplicate). This

value was close to that obtained by calculation with ACD/Labs software (logD = 1.84).

3.3. In vitro distribution in rat brain

In vitro distribution of [^{11}C]PF-3274167 was evaluated by autoradiography on rat brain slices (Fig. 2). Overall, there were very few differences of radioactivity level among brain regions with the standard washing conditions (Fig. 2A). The highest binding was found in the cerebellum. A slightly higher binding was also observed in the hippocampus and the striatum. By changing the washing conditions (adding ethanol to the buffer), the distribution of the tracer matched with the white matter distribution but was almost absent from the rest of the brain (Fig. 2B).

3.4. In vitro competition studies

In all regions of interest, [^{11}C]PF-3274167 binding was unchanged by the addition of increasing concentrations of cold PF-3274167 (Fig. 3A) or carbetocin, an OTR agonist, from 1 nM to 100 nM (Fig. 3B).

3.5. In vivo distribution in rat brain

In vivo distribution of [^{11}C]PF-3274167 was visualized using microPET in isoflurane-anaesthetized rats. In control conditions, the brain uptake was very low compared to the rest of the body: below 0.5 SUV at the beginning of the scan, compared to 2.5 SUVs in the heart (Fig. 4A and 4B). In the rat that received a ciclosporin pre-injection to inhibit P-gp activity, the brain penetration of [^{11}C]PF-3274167 was clearly increased to a radioactivity level quantifiable by the PET camera (Fig. 4C), with a peak reached between 10 and 15 min and a fast decrease during the rest of acquisition (Fig. 4D). However, the time–activity curves showed modest differences between brain regions (between 1.2 and 1.7 SUVs at 15 min after injection), with the highest uptake in brainstem, striatum, thalamus and hypothalamus, and the lowest uptake in the occipital cortex. In the rat pre-injected with WAY-267,464 in addition to ciclosporin, distribution pattern and uptake were similar (between 1.4 and 1.8 SUVs at 15 min after injection).

4. Discussion

Currently, there is no validated radiotracer available for PET imaging of the oxytocin receptor *in vivo*. In this context we chose to radiolabel the specific antagonist PF-3274167, which was previously identified by a Pfizer research group [14]. This compound would present several advantages as a PET tracer. First, it possesses good affinity for the oxytocin receptor ($K_i = 9.5\text{ nM}$). Second, it has good selectivity over the vasopressin receptors (>100 -fold), which is a clear advantage taking into account the structural similarities between the oxytocin and the vasopressin receptors. Third, PF-3274167 was also shown to be able to cross a simplified model of the blood–brain barrier *in vitro* [15]. Fourth, the compound should be easy to radiolabel with ^{11}C on the O-methyl position.

The radiolabeling of the desmethyl precursor **1** whose synthesis was previously described [16] was straightforward, and [^{11}C]PF-3274167 was produced with a good radiochemical yield, high radiochemical and chemical purity, and a specific activity suitable for PET imaging.

We performed autoradiography experiments to evaluate *in vitro* binding of [^{11}C]PF-3274167 in rat brain. Unfortunately, the autoradiograms revealed barely detectable differences between the OTR-rich regions, such as ventral subiculum, ventral pallidum and lateral septum in adult male rats [21,22] and the OTR-poor regions like cingulate cortex and thalamus. Furthermore, the highest binding was observed in some parts of the cerebellum, which is not a region usually described as expressing the oxytocin receptor. Therefore, this unexpected *in vitro*

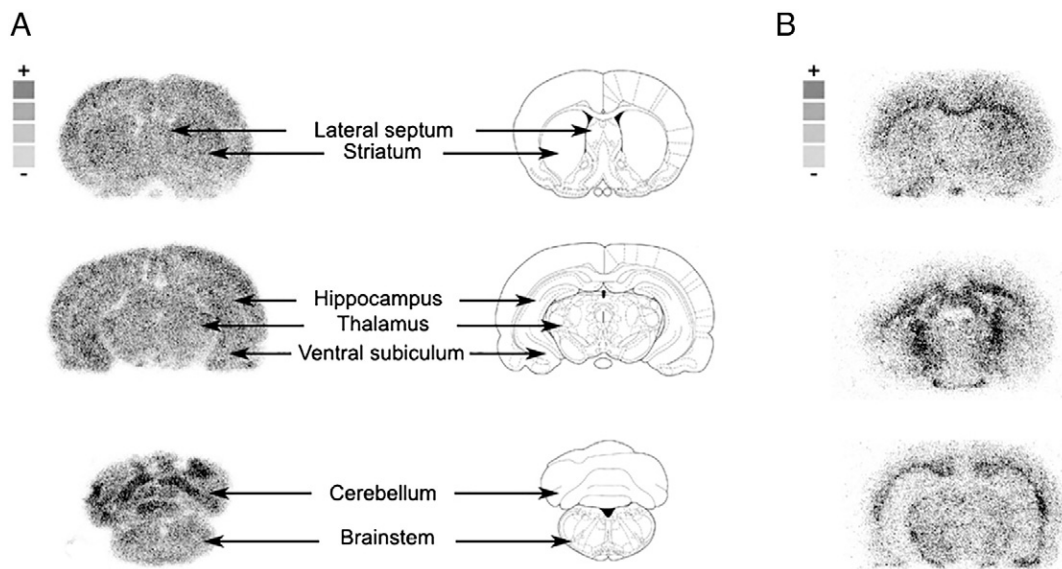


Fig. 2. **A.** In vitro autoradiograms of rat brain sections incubated with [¹¹C]PF-3274167 and corresponding anatomic slices (Paxinos and Watson, 1986). **B.** In vitro autoradiograms of rat brain sections incubated with [¹¹C]PF-3274167 and rinsed with TBS supplemented with ethanol, showing the strong interaction with white matter.

distribution was likely to reflect non-specific binding. This was confirmed by the absence of decrease of radioactivity after the addition of cold PF-3274167 or carbetocin in the incubation bath, even at the highest concentrations. The non-specific binding is likely to be explained by a non-optimal affinity of [¹¹C]PF-3274167 for the oxytocin receptor ($K_i = 9.5$ nM). In vivo, brain density of oxytocin receptors has not been documented at this time because of the lack of a suitable radiotracer; in vitro, B_{max} values of 20 fmol/mg of protein have been

reported in amygdala of females rats with [¹²⁵I]OTA [23]. Considering this low density of receptors, a radiotracer with a subnanomolar affinity would be required, as a B_{max}/K_d ratio value of at least 4 is advised for PET imaging [24] and a higher ratio value for compounds with non-specific binding [25]. It should also be taken into account that the incubation time in autoradiography experiments should be long enough to reach equilibrium, and might be too short in our study because of the short half-life of ¹¹C. We also tried to change the rinsing conditions by adding

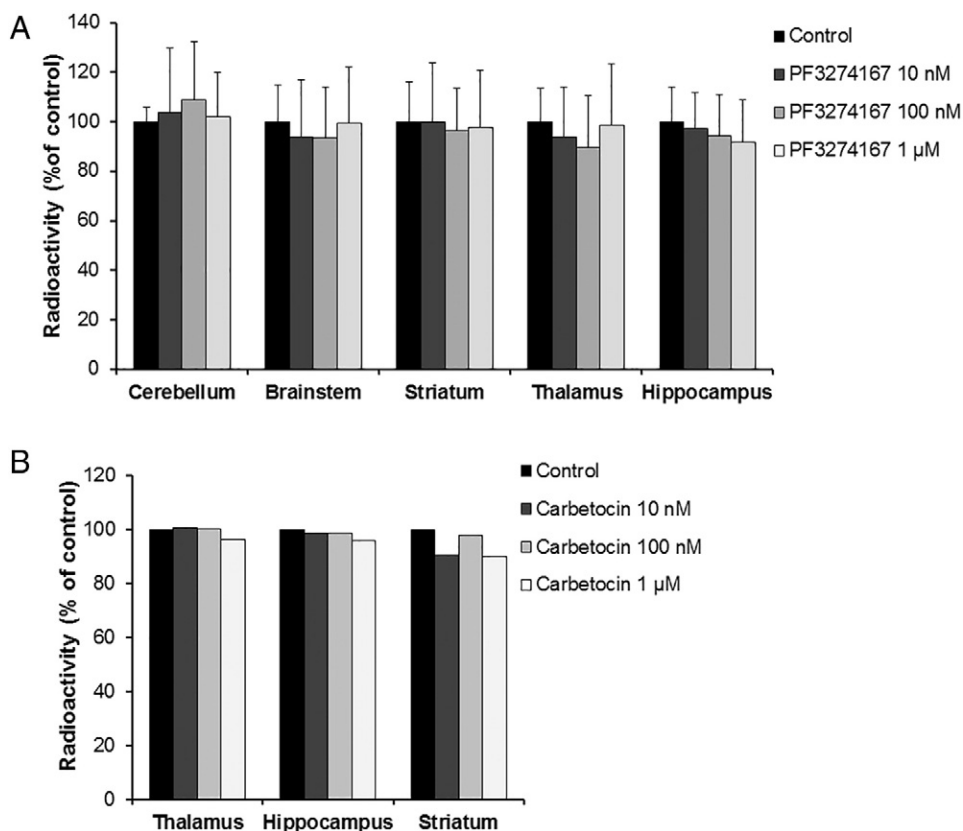


Fig. 3. **A.** In vitro competition in rat brain between [¹¹C]PF-3274167 and unlabeled PF-3274167 at increasing concentrations. **B.** In vitro competition in rat brain between [¹¹C]PF-3274167 and carbetocin at increasing concentrations.

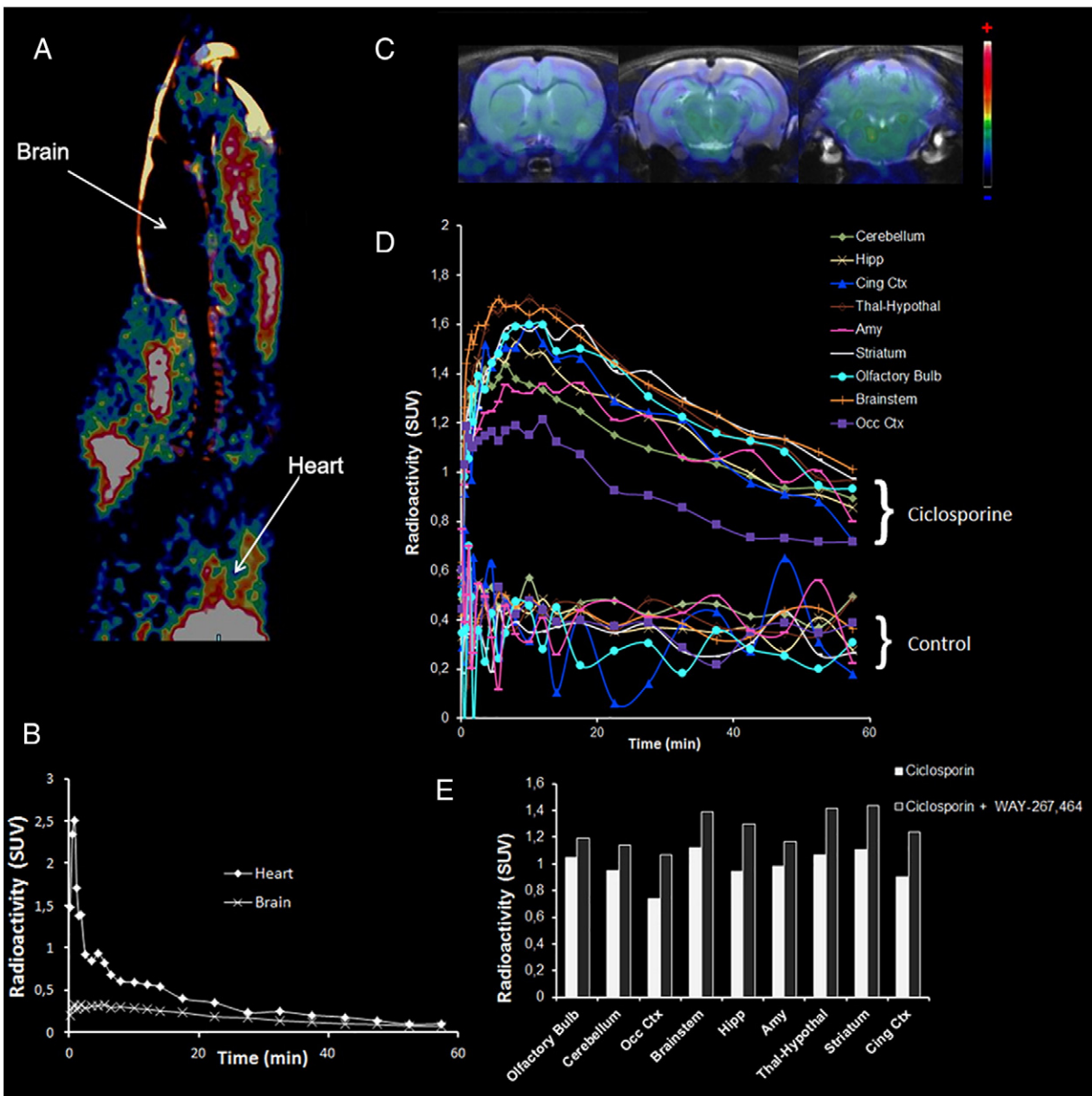


Fig. 4. In vivo distribution of [^{11}C]PF-3274167 in isoflurane-anesthetized rat. **A.** Sagittal microPET image summed for 60 minutes superposed with the CT image acquired in the same scan, showing [^{11}C]PF-3274167 distribution in rat in vivo. **B.** Time-activity curves of [^{11}C]PF-3274167 in a rat brain and heart. **C.** Coronal microPET images summed for 60 minutes on corresponding MRI images, showing [^{11}C]PF-3274167 distribution in a rat brain pretreated with cyclosporin at 50 mg/kg intravenously, 30 minutes before the scan. **D.** Time-activity curves of [^{11}C]PF-3274167 in several regions of interest in a rat brain. Bottom of the graph: the rat was pretreated with saline; top of the graph: the same rat was pretreated with cyclosporin. Hipp = Hippocampus; Cing Ctx = Cingulate cortex; Thal-Hypothal = Thalamus and hypothalamus; Amy = Amygdala; Occ Ctx = Occipital cortex. **E.** [^{11}C]PF-3274167 SUV mean values between 20 and 60 minutes of acquisition in several regions of interest; comparison between a rat pretreated with cyclosporin at 50 mg/kg i.v. 30 minutes before the scan (in white) and a rat pretreated with cyclosporin (same conditions) and WAY-267,464 at 30 mg/kg i.p. 20 minutes before the scan (in grey). The dose injected in the scan corresponding to figures A and B was 37 KBq/g. The dose injected in the scan corresponding to figures C, top of D and E (in white) was 43 KBq/g. The dose injected in the scan corresponding to the bottom of D was 25 KBq/g. The dose injected in the scan corresponding to figure E (in grey) was 29 KBq/g.

ethanol in order to reduce non-specific binding, which resulted in autoradiograms where binding was high in white matter only suggesting that non-specific binding is also due to strong interactions between [^{11}C]PF-3274167 and myelin. This characteristic was unexpected, based on the measured logD value of 1.93 (i.e. at physiological pH), meaning that the compound is not highly lipophilic. The possible influence of impurities on this experimental value is unlikely because of its similarity with the predicted logD value. In any case, [^{11}C]PF-3274167 lipophilicity is in a desirable range, in comparison to other common PET brain radiotracers [26]. It is more plausible that the compound is able to bind to the beta-sheet structure of myelin constituents, due to its heterocyclic flat part, similarly to ^{18}F -florbetaben or ^{11}C -PIB, both showing high uptake in white matter [27,28].

Finally, we evaluated the distribution of [^{11}C]PF-3274167 in rat brain *in vivo* using microPET imaging. Similarly to previous radioligands tested [8–11] and despite the encouraging *in vitro* data of the molecule [15], the images clearly revealed a lack of [^{11}C]PF-3274167 brain delivery. This kind of failure can usually be explained by several factors, such as inadequate lipophilicity or active efflux from the brain by the P-glycoprotein that is known to strongly regulate brain uptake of drugs [26,29]. As a logD of 1.93 is compatible with a good brain penetrance [26], we hypothesized that [^{11}C]PF-3274167 was expelled from the brain by P-gp. Therefore, we performed another microPET acquisition with a rat pretreated with cyclosporin that has been shown to inhibit P-gp activity [30] and brain radioactivity was clearly increased to quantifiable levels, indicating that [^{11}C]PF-3274167 is indeed a P-gp

substrate. However, the distribution pattern showed no clear differences in radioactivity levels between the OTR-rich regions and the OTR-poor regions (like, for instance, hippocampus versus cingulate cortex), indicating that [¹¹C]PF-3274167 binding *in vivo* is likely to be non-specific similarly to the *in vitro* results. We performed a blocking experiment with WAY-267,464, a non-peptide oxytocin receptor agonist that has central effects in rodents when injected intraperitoneally at high doses [31]. The radiotracer distribution pattern in the rat injected with WAY-267,464 at 30 mg/kg i.p., in addition to ciclosporin, was similar to the rat treated with ciclosporin alone. Furthermore, no blocking effect was observed in any region of interest and global brain uptake was even slightly higher, confirming the lack of specific binding.

The overall results tend to show that [¹¹C]PF-3274167 is not suitable for imaging of the oxytocin receptor. We explain this failure mainly by a suboptimal affinity, and we plan on evaluating future candidates on other species in addition to rat, considering that the influence of P-gp on uptake of drugs in the central nervous system can vary significantly between species [32].

5. Conclusion

We report the radiosynthesis and the preclinical evaluation of the specific antagonist [¹¹C]PF-3274167 as a radiotracer for PET neuroimaging of the central oxytocin receptor. Despite promising *in vitro* properties and an easy radiolabeling, the candidate failed to show any evidence of specific binding in rat brain, both *in vitro* and *in vivo*. [¹¹C]PF-3274167 also seems to be a P-gp substrate in rat, resulting in a lack of brain uptake in basal conditions. [¹¹C]PF-3274167 is therefore not suitable for mapping the oxytocin receptor. Further investigations should focus on other candidates with a higher affinity for OTR.

Acknowledgements

This research was funded by the French National Research Agency (project# ANR-14-CE16-0005-03). This work was performed within the framework of the LABEX MEDALIS of the University of Strasbourg and the framework of the LABEX PRIMES of Université de Lyon, both operated by the French National Research Agency (ANR).

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