Am J Nucl Med Mol Imaging 2018;8(1):32-40
www.ajnmmi.us /ISSN:2160-8407/ajnmmi0069681

Original Article

Synthesis, radiolabelling, and evaluation of [^{11}C]PB212 as a radioligand for imaging sigma-1 receptors using PET

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Received November 23, 2017; Accepted December 20, 2017; Epub February 5, 2018; Published February 15, 2018

Abstract: The Sigma-1 receptor (Sig-1R) has been described as a *pluripotent modulator* of distinct physiological functions and its involvement in various central and peripheral pathological disorders has been demonstrated. However, further investigations are required to understand the complex role of the Sig-1R as a molecular chaperon. A specific PET radioligand would provide a powerful tool in Sig-1R related studies. As part of our efforts to develop a Sig-1R PET radioligand that shows antagonistic properties, we investigated the suitability of 1-(4-(6-methoxynaphthalen-1-yl)butyl)-4-methylpiperidine (designated PB212) for imaging Sig-1R. PB212 is a Sig-1R antagonist and exhibits subnanomolar affinity (K_i = 0.030 nM) towards Sig-1R as well as good to excellent selectivity over Sig-2R. The radiolabelling of [^{11}C]PB212 was accomplished by *O*-methylation of the phenolic precursor using [^{11}C]MeI. In vitro autoradiography with [^{11}C]PB212 on WT and Sig-1R KO mouse brain tissues revealed high non-specific binding, however using rat spleen tissues from CD1 mice and Wistar rats, high specific binding was observed. The spleen is known to have a high expression of Sig-1R. In vivo PET experiments in Wistar rats also showed high accumulation of [^{11}C]PB212 in the spleen. Injection of Sig-1R binding compounds, haloperidol (1 mg/kg) or fluspidine (1 mg/kg) shortly before [^{11}C]PB212 administration induced a drastic reduction of radiotracer accumulation, confirming the specificity of [^{11}C]PB212 towards Sig-1R in the spleen. The results obtained herein indicate that although [^{11}C]PB212 is not suitable for imaging Sig-1R in the brain, it is a promising candidate for the detection and quantification of Sig-1Rs in the periphery.

Keywords: PB212, sigma-1 receptor, PET imaging

Introduction

The term sigma receptor was initially introduced by Martin and colleagues in 1976 to describe the target of the psychotomimetic benzomorphan SKF-10,047 (*N*-allylnormetazocine) [1]. This receptor was first included in the opioid system (sigma *opioid* receptor) since the effects of SKF-10,047 were blocked by the opioid antagonist naltrexone [1]. However, subsequent studies revealed that the SKF-10,047 binding site was poorly inhibited by naltrexone and that the psychotomimetic effects of this drug in dogs were not antagonized by naltrexone pretreatment [2, 3]. Hence, the sigma *opioid* receptor introduced by Martin emerged as a non-opioid, non-phencyclidine, and non-dopamine receptor so that the term 'opioid' was removed from the definition of the sigma receptor (Sig-R) [4]. To date, we know that the sigma system consists of at least two different receptor subtypes, Sigma-1 (Sig-1R) and Sigma-2 (Sig-2R), differing in molecular weight, localization, ligand engagement as well as biological functions [5, 6].

The Sig-1R was cloned in 1996, yielding a protein of 223 amino acids which exhibits no homology with any other known mammalian protein [7, 8]. Several non-related endogenous compounds, such as steroids (e.g., progesterone, dehydroepiandrosterone sulfate, etc.), the

hallucinogen *N,N*-dimethyltryptamine, and sphingosine have been proposed as endogenous ligands of Sig-1R. However, their affinities are not sufficient to explicitly define their role as endogenous ligands [4, 9-12]. Furthermore, Sig-1R shows moderate to high affinity towards a wide spectrum of exogenous ligands, thereby covering several different structural classes and with distinct pharmacological effects (neuroleptics, antidepressants, antitussives, drugs for the treatment of neurodegenerative disorders, drugs of abuse, etc.) [9]. Sig-1Rs are widely distributed in the human body, both in the central nervous system (CNS) [13, 14] and in the periphery (mainly in spleen, liver, lung, kidney, and adrenal gland) [6, 7, 14-17]. At the subcellular level, the Sig-1R is mainly localized at the endoplasmic reticulum, specifically at the interface with the mitochondrion, but it is also found in the plasma membrane and in the nuclear envelope [9, 18]. The information available on the physiology and the pharmacology of Sig-1Rs supports their classification as molecular chaperons, regulating the activity of several cellular proteins, such as receptors, ion channels, kinases, etc. [9, 18, 19].

Taken together, the wide anatomical and subcellular distribution, the ability to interact with several different exogenous ligands, as well as the chaperon activity towards different cellular targets render the Sig-1R a *pluripotent modulator* [18] involved in many physiological pathways and, consequently, in many central and peripheral pathological disorders (comprehensive reviews: Cobos et al. [9], Su et al. [18], Maurice and Su [19], Nguyen et al. [20]). However, further investigations are crucial to understand the complex role of Sig-1Rs both in physiological and pathological conditions. Hence, the development of specific radiotracers for PET imaging provides a powerful tool in this direction. In this study, we aim to evaluate PB212 (1-(4-(6-methoxynaphthalen-1-yl)butyl)-4-methylpiperidine) [21] as a potential PET imaging agent for Sig-1R. PB212 exhibits subnanomolar affinity towards Sig-1R (K_i = 0.030 nM) [21], good to excellent selectivities against Sig-2R (K_i = 17.9 nM) [21], emopamil binding protein (EBP, K_i = 8.04 nM) [21], serotonin (K_i 5-HT_{1A} > 1000 nM, K_i 5-HT_7 > 1000 nM), dopamine (K_i D2R > 1000 nM, K_i D3R > 1000 nM) and adrenergic receptors (IC_{50} α_1 > 100 nM) [22]. In addition, PB212 has shown antagonistic properties both in in vitro [23] and in vivo [22] assays. Almost all Sig-1R radiotracers developed to date are agonists and as such comparison of the data obtained from [^{11}C]PB212 with Sig-1R agonists, such as [^{11}C]SA4503, could potentially provide important information about Sig-1R physiology, given that agonists and antagonists may bind to different states of the receptor. With this in mind, we radiolabelled PB212 with carbon-11 and assessed its suitability as a PET radioligand for imaging Sig-1R [24].

Materials and methods

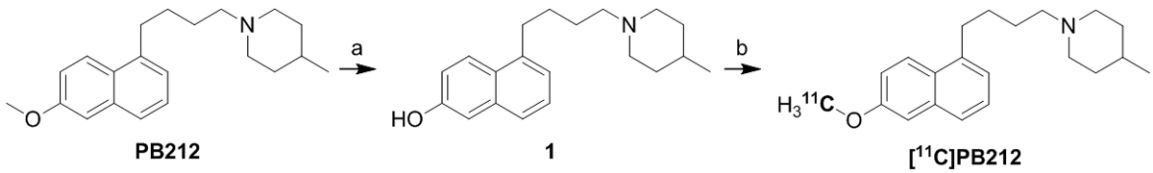
Animal experiments were conducted in accordance with the Swiss Animal Welfare legislation and were approved by the Veterinary Office of the Canton Zurich. Male Wistar rats were purchased from Charles River (Sulzfeld, Germany) and kept under standard conditions.

All chemicals, unless otherwise stated, were purchased from Sigma Aldrich GmbH (Taufkirchen, Germany), ABCR GmbH (Karlsruhe, Germany), Merck (Darmstadt, Germany), or Fluka (Buchs, Switzerland) and were used without further purification. Fluspidine was kindly provided by Prof. Bernand Wünsch (Westfälische Wilhelms-Universität Münster, Germany).

Analytical radio-HPLC was performed with a flow rate of 1 mL/min on an Agilent 1100 series system equipped with a Raytest Gabi Star radiodetector (Agilent Technologies, Morges, Switzerland). Semi-preparative HPLC purifications were carried out using a reversed phase column (Phenomenex Luna, 5 µm, 250 × 10 mm) under the following conditions: 50 mM ammonium formate in H_2O pH = 4.4 (solvent A), MeCN (solvent B); isocratic, 45% B; flow rate: 5 mL/min. Molar activity was calculated by comparing ultraviolet peak intensity of the final formulated product with calibration curve of corresponding non-radioactive standard of known concentrations.

Radiochemistry

[^{11}C]CO_2 was produced by proton bombardment of nitrogen gas fortified with 0.5% oxygen using IBA Cyclone 18/9 cyclotron (18-MeV; IBA, Ottignies-Louvain-la-Neuve, Belgium) applying the well established ^{14}N(p,a)^{11}C nuclear reaction. In a first step, nickel-based catalytic reduction of [^{11}C]CO_2 yielded [^{11}C]CH_4 which was subsequently iodinated to give [^{11}C]MeI. Subse-



Scheme 1. Synthesis of precursor 1 [25] and radiosynthesis of [^{11}C]PB212. a) BBr_3, dry CH_2Cl_2, 78°C→rt; b) [^{11}C]CH_3I, Cs_2CO_3, dry DMF.

quently, [^{11}C]MeI was bubbled into a reaction mixture containing phenolic intermediate 1 (1 mg) and cesium carbonate (6 mg) in DMF (0.4 mL) and stirred at 90°C for 3 min. After dilution of the crude product with water (1.6 mL), purification was performed by semi-preparative HPLC. The collected radiotracer was diluted with water (8 mL) and passed through a C18 cartridge (Waters, pre-conditioned with 5 mL EtOH and 10 mL water). After washing of the cartridge with water (5 mL), the product was eluted with EtOH (0.8 mL) into a sterile vial and diluted with water for injection (9.2 mL) to give a final formulation containing 8% of ethanol. For quality control, an aliquot of the final formulation was injected into the analytical HPLC system. Identity of the product was confirmed by co-injection and comparison with the retention time of the standard reference. The molar activity was calculated by linear regression using a UV-intensity based calibration curve of standard reference.

In vitro autoradiography

Autoradiography was performed on rat and mouse brain as well as spleen tissues. Sections were prepared in 10 µm thickness using a cryostat (Cryo-StarNX50; Thermo Scientific). The tissue slices were adsorbed to SuperFrost Plus (Menzel) slides and stored at -20°C until use. After thawing at room temperature (rt) for 10 min, sections were pre-incubated for 15 min at rt in the incubation buffer (50 mM TRIS·HCl and 0.01% BSA in H_2O; pH 7.4). Slices were dried and incubated in a humidified chamber with [^{11}C]PB212 (0.3-0.5 nM) in incubation buffer for 15 min at rt. To test for specificity towards Sig-1R, solutions containing the radiotracer and an excess of a different Sig-1R ligand (either haloperidol, SA4503, or fluspidine; 10 µM) were prepared and added to the tissues. The tissue slices were washed three times with the washing buffer (50 mM TRIS·HCl in H_2O; pH 7.4) (each 2 min) and twice with distilled water (each 5 s) on ice, air dried, and exposed to a phosphor imager plate for a period of 30 min. The plate was scanned using a BAS5000 reader (Fujifilm, Dielsdorf, CH).

In vivo PET/CT imaging

PET and CT scans were obtained with a Super Argus PET/CT tomograph (Sedecal, Madrid, Spain) after injection of [^{11}C]PB212 (18.1-21.4 MBq, 0.58-1.36 nmol/kg) into the tail of male Wistar rats (357-389 g, n = 3) which were kept under anesthesia using isoflurane. Under baseline conditions, radiotracer accumulation was recorded in the region of the spleen in dynamic PET acquisition mode over 90 min. During this period body temperature and respiratory rate were constantly monitored. Under blockade conditions, 1 mg/kg of either haloperidol or fluspidine were injected 30 seconds before radiotracer application in two of the three rats. Acquired PET data were reconstructed as user-defined time frames with a voxel size of 0.3875 × 0.3875 × 0.775 mm. For anatomical orientation, CT scans were acquired after each PET scan. Images were evaluated with PMOD v3.4 (PMOD Technologies Inc., Zurich, CH) software. Regions of interest (spleen and muscle) were drawn manually using the PMOD fusion tool. Time activity curves (TACs) for spleen and muscle were expressed as standardized uptake values (SUVs).

Results

Radiochemistry

Reference compound PB212 was synthesized according to a published procedure [21] and subsequently demethylated by reaction with BBr_3 to provide phenolic precursor 1 [25]. The radiosynthesis of [^{11}C]PB212 was accomplished by *O*-methylation of the cesium salt of phenolic precursor 1 using [^{11}C]MeI (**Scheme 1**). The obtained radiochemical yields ranged from 16 to 33% (decay corrected) with molar

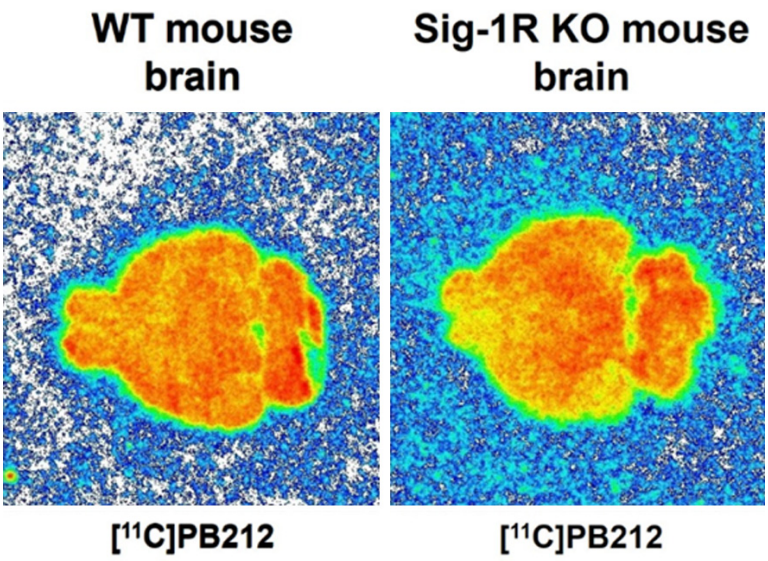


Figure 1. In vitro autoradiography with [11C]PB212 on WT and Sig-1R KO mouse brain tissues.

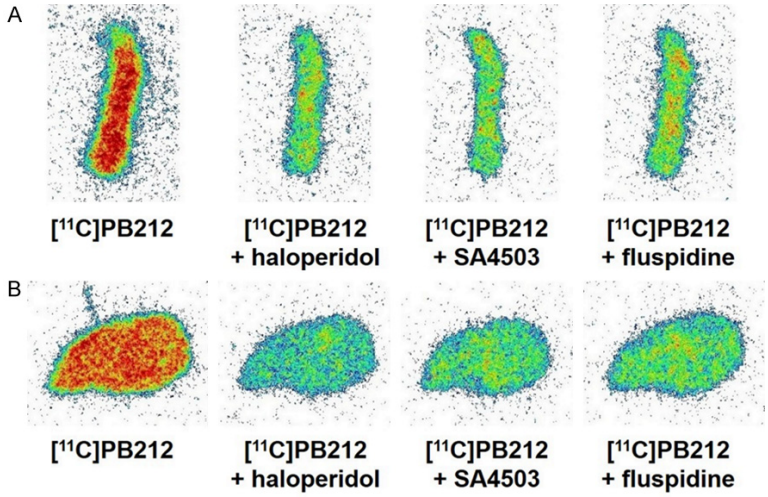


Figure 2. In vitro autoradiography with [11C]PB212 on mouse (A) and rat (B) spleen tissues. For blocking conditions, an excess (10 μM) of either haloperidol, SA4503, or fluspidine was used.

activities ranging between 39 and 391 GBq/μmol at the end of synthesis. In all cases, a radiochemical purity ≥ 99% was obtained after semi-preparative HPLC purification. The total radiosynthesis time from the end of bombardment to the end of synthesis was approximately 30 min.

In vitro characterization

In vitro autoradiography with WT and Sig-1R KO mouse brain tissues revealed high binding of [11C]PB212 in both wildtype and Sig-1R KO mouse, which could not be blocked in wild type mouse, indicating that the binding to mouse brain tissue is mainly nonspecific (**Figure 1**). In contrast, high and specific binding of [11C]PB212 was observed in the autoradiography experiments using spleen tissues obtained from CD1 mice (**Figure 2A**) and Wistar rats (**Figure 2B**). The high and known physiological expression of Sig-1R in the spleen prompted us to use the spleen as a target organ for the evaluation of [11C]PB212 for imaging Sig-1R expression in the periphery [14-16]. Three different blockers were selected and used to assess [11C]PB212 binding specificity towards Sig-1Rs in the spleen: 1) haloperidol, a nonselective Sig-1R ligand (K_i Sig-1R = 0.9 nM, $SI_{Sig\text{-}2R/Sig\text{-}1R}$ = 8.8) [26] with high binding affinity also for dopamine receptors (e.g., K_i D2R = 2.0 nM, K_i D3R = 4.0 nM, K_i D4R = 15 nM) [27], serotonin receptors (e.g., K_i 5-HT_{2A} = 70 nM) [27], and adrenergic receptors (e.g., K_i α_1 = 12 nM) [27]; 2) SA4503, a Sig-1R ligand (K_i Sig-1R = 4.63 nM, $SI_{Sig\text{-}2R/Sig\text{-}1R}$ = 13.6) [26] with weak or no binding towards several receptors, ion channel, and second messenger systems [28], except for the EBP (K_i = 1.7 nM) [29] and the vesicular acetylcholine transporter (VAChT, K_i = 50.2 nM) [30]; 3) fluspidine, a selective Sig-1R ligand (K_i Sig-1R = 0.59 nM, $SI_{Sig\text{-}2R/Sig\text{-}1R}$ = 1331) [31] with weak binding affinities towards several receptors (e.g., phencyclidine NMDA binding site, μ, δ, and κ receptors) [31], including EBP (K_i = 211 nM) and VAChT (K_i = 1.4 μM) [15].

In vivo characterization

Time activity curves (TACs) for spleen and muscle under baseline and blockade conditions are depicted in **Figure 3**. Blocking experiments

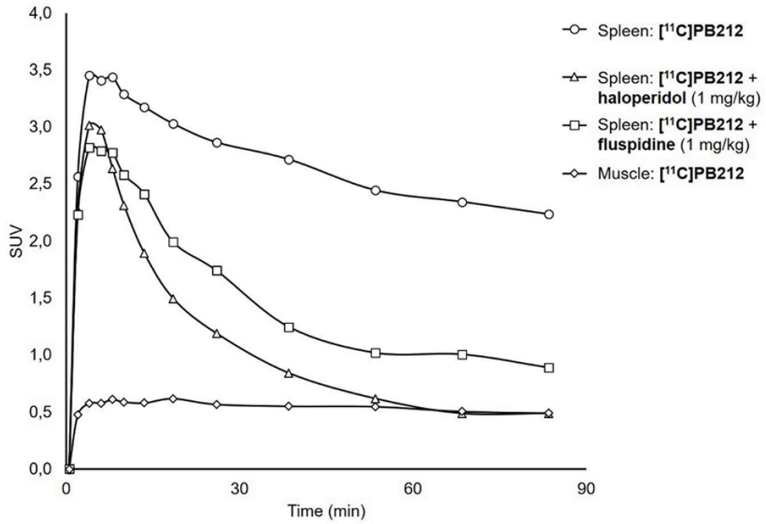


Figure 3. Typical time activity curves of [^{11}C]PB212 in whole spleen and muscle under baseline or blocking conditions with 1 mg/kg of either haloperidol or fluspidine shortly prior injection of the radioligand.

were performed using either haloperidol or fluspidine because of their different binding profile: the former is a nonselective Sig-1R ligand, and the latter can be considered one of the most selective Sig-1R ligands currently available. The injection of haloperidol (1 mg/kg) shortly before [^{11}C]PB212 administration led to a drastic reduction of the radiotracer accumulation in the spleen, reaching radioactivity levels detected in the muscle (background region) 60 minutes post injection. The injection of fluspidine (1 mg/kg) induced a partial, but significant, reduction of [^{11}C]PB212 accumulation in the spleen.

Discussion

Sig-1Rs play a crucial but not yet fully understood role in physiological and pathological conditions. A connection of Sig-1R with cocaine abuse has been demonstrated [32, 33], while knocking-down of Sig-1R has been associated with neurotoxic effects and neurodegeneration in amyotrophic lateral sclerosis (ALS), Alzheimer's Disease (AD), Parkinson's Disease (PD), and Huntington's Disease (HD). Additionally, some Sig-1R ligands have shown important therapeutic effects in the animal models of these neurodegenerative diseases [18, 20]. Accordingly, clinical trials are currently ongoing to evaluate the diagnostic and therapeutic potentials of Sig-1R ligands [34, 35]. Furthermore, Sig-1R overexpression has been found in several tumor cell lines and tumor biopsies, and anti-cancer effects have been observed for some Sig-1R ligands [36-38]. A specific Sig-1R PET tracer would help to gain deeper insight into the physiological and pathological pathways in which the Sig-1R is involved, both in the CNS and in the periphery. In addition, such a tracer could be a useful tool not only for detecting the down- or upregulation of Sig-1Rs associated with different disorders, but also for the monitoring of disease progression and therapeutic outcome in the clinic.

Several radioligands have been developed for the PET imaging of Sig-1R and a summary of existing ligands can be found in [39-42]. [^{11}C]SA4503 [16], [^{18}F]fluspidine [15] and [^{18}F]FTC-146 [43] are three examples of the most assessed radioligands for PET imaging of Sig-1R in the CNS. [^{11}C]SA4503 is one of the first valuable radiotracers developed for brain PET imaging of Sig-1R and it has been studied in healthy human volunteers as well as in PD and AD patients [44, 45]. The radioligands with longer half-lives [^{18}F]fluspidine and [^{18}F]FTC-146 have been tested in human volunteers and have emerged as two promising ^{18}F-radiolabelled Sig-1R radiotracers for brain PET imaging [46, 47]. Much more limited is the development of radioligands for PET detection of Sig-1R in cancer, with [^{11}C]SA4503 showing specific uptake in tumor-bearing rodents [48-52]. Nevertheless, the clinical utility of these radiotracers has not yet been established. In this study, we therefore aimed to evaluate [^{11}C]PB212, which is an antagonist and shows high affinity for Sig-1R and selectivity towards other receptors. In particular, the selectivity of PB212 over Sig-2R receptor subtype is much higher than the selectivity reported for SA4503 [21, 22, 26].

The ^{11}C-radiolabelling was successfully achieved using [^{11}C]MeI as methylating agent leading to high molar activity and ≥ 99% radiochemical purity of the final product. In vitro autoradiography experiments showed that [^{11}C]PB212 binds homogenously to WT and Sig-1R knock-out mouse brain, demonstrating a lack of tissue

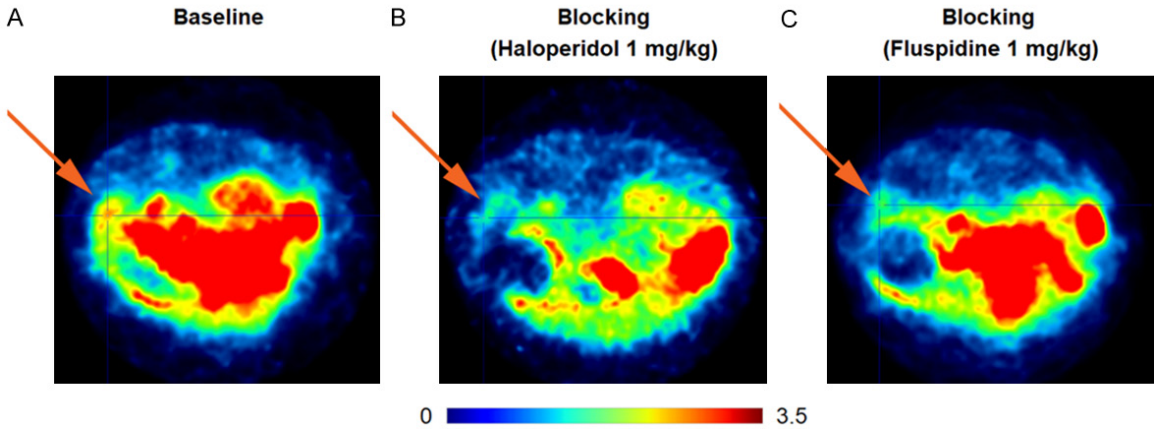


Figure 4. Transaxial PET images of the abdominal region, averaged from 0 to 60 minutes post injection. The spleen is indicated by an arrow. After injection of the tracer (18-21 MBq), the animals were maintained at 2% isoflurane for anesthesia. Color bar indicates SUV. A: Baseline conditions, B: Blockade conditions using haloperidol (1 mg/kg), C: Blockade conditions using fluspidine (1 mg/kg).

specificity (**Figure 1**). This result is in agreement with previous reports, which showed that picomolar affinities lead to low specific binding of Sig-1R radiotracers to brain tissue [40]. In addition, we speculate that the lipophilicity of [^{11}C]PB212 (clog$D_{7.4}$ = 2.38, calculated through https://chemicalize.com/) could be a reason for the lack of CNS specificity. Nevertheless, in vitro autoradiography in rodent spleen tissue demonstrated a complete displacement of [^{11}C]PB212 in the presence of three Sig-1R blockers (i.e. haloperidol, SA4503 and fluspidine, **Figure 2A**, **2B**). These results were further confirmed by in vivo PET imaging in Wistar rats. **Figure 3** shows the corresponding TACs comparing [^{11}C]PB212 uptake in the spleen and background (muscle) under baseline conditions with blockade conditions using either haloperidol or fluspidine. PET images are presented in **Figure 4** and show the tracer accumulation in the spleen under baseline (panel A) as well as blockade conditions with haloperidol (panel B) and fluspidine (panel C). Both haloperidol and fluspidine administered at a concentration of 1 mg/kg significantly reduced radioactivity uptake in the spleen, suggesting reversible and specific binding of [^{11}C]PB212 to Sig-1R. The reduction of radioactivity in the spleen was more pronounced with haloperidol and reached background (muscle) levels towards the end of the studies.

The results of the PET imaging studies in the spleen clearly demonstrate that [^{11}C]PB212 can be used to assess the peripheral expression of Sig-1R in vivo and support the further evaluation of [^{11}C]PB212 in the periphery.

Conclusion

Here, we describe the radiolabelling and the biological evaluation of [^{11}C]PB212, a well described Sig-1R antagonist with subnanomolar affinity and excellent selectivities against a number of receptors. [^{11}C]PB212 was synthesized in good radiochemical yields (16-33%) and excellent radiochemical purity as well as good molar activity (39-391 GBq/μmol). Although [^{11}C]PB212 is not suitable for imaging Sig-1R in the brain, the high in vitro and in vivo specificity observed in the spleen suggests that [^{11}C]PB212 can be used to image Sig-1R expression in the periphery. These promising results warrant further studies in Sig-1R-positive tumor bearing mice in order to shed more light on the utility of [^{11}C]PB212 for peripheral Sig-1R PET detection in healthy and diseased organs. Furthermore, being a Sig-1R antagonist, [^{11}C]PB212 could provide additional information on Sig-1R physiology which cannot otherwise be obtained with Sig-1R agonists such as [^{11}C]SA4503 and [^{18}F]fluspidine.

Acknowledgements

We thank Mr. Bruno Mancosu for his support with carbon-11 radiolabelling, and Prof. Bernard Wünsch (Department of Pharmaceutical and Medicinal Chemistry, Westfälische Wilhelms-Universität Münster, Germany) for kindly providing fluspidine.

Disclosure of conflict of interest

None.

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