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Evaluation of $[^{18}\text{F}]$Fluoromethyl PBR28 as a Translocator Protein (18 kDa) PET Ligand for Imaging Neuroinflammation

TSPO PET 방사성추적자 $[^{18}\text{F}]$Fluoromethyl PBR28 을 이용한 신경염증영상 연구

2013年 8月

서울대학교 대학원
의학과 뇌신경과학 전공

김 범 산
Abstract

Evaluation of $[^{18}\text{F}]$Fluoromethyl PBR28 as a Translocator Protein (18 kDa) PET Ligand for Imaging Neuroinflammation

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Purpose: The neurodegenerative condition with neuronal loss was well correlated with increased upregulation of the translocator protein (18 kDa) (TSPO), which was formerly known as the peripheral benzodiazepine receptor by activated microglia. Here, we detail the synthesis of a newly developed $[^{18}\text{F}]$fluoromethyl-PBR28 ($N$-acetyl-$N$-(2-$[^{18}\text{F}]$fluoromethoxybenzyl)-2-phenoxy-5-pyridinamine) as a derivative
of PBR28 ([¹⁸F]FM-PBR28) and evaluate the suitability of [¹⁸F]FM-PBR28 as a biomarker of neuroinflammatory disease.

**Materials and Methods:** [¹⁸F]FM-PBR28 was prepared from nucleophilic aliphatic substitution on the triazolium triflate precursor of the PBR28 precursor with fluorine-18 in a single-step radiolabeling procedure. [¹¹C]PBR28 was produced in FX C pro module using [¹¹C]MeOTf from the phenolic PBR28 precursor according to the literature. The TSPO binding affinity and lipophilicity were measured by competition with [³H]PK11195 in the membrane of a human leukocyte and the octanol-buffer partition coefficient, respectively (n=4). Four days after injection of lipopolysaccharide (LPS) in the ipsilateral striatum, neuroinflammatory positron emission tomography (PET) studies using [¹⁸F]FM-PBR28 and [¹¹C]PBR28 were performed (n=5). Additional neuroinflammatory PET studies involving the displacement with unlabeled PBR28 (5 mg/kg, n=4), and the co-injection with unlabeled FM-PBR28 (5 mg/kg, n=3), and flumazenil (5 mg/kg, n=3) were performed to evaluate the specificity and selectivity of [¹⁸F]FM-PBR28.

**Results:** [¹⁸F]FM-PBR28 has been efficiently synthesized in 25.8 ± 3.2% radiochemical yield. The final pure [¹⁸F]FM-PBR28 was purified by a reverse-phase HPLC system, and the collected solution was reformulated with 5% EtOH/saline using a tC18 Sep-Pak cartridge with a radiochemical purity of over 99%. [¹¹C]PBR28 was produced in 27.1 ± 4.9% of radiochemical yield based on [¹¹C]CO₂. FM-PBR28 and PBR28 exhibited similar in vitro binding affinity (IC₅₀) and log D values (8.28 versus 8.07 nM and 2.85 versus 3.01, respectively).
In a neuroinflammatory rat model, an over 3.4 higher level of uptake of $[^{18}F]$FM-PBR28 than that of contralateral striatum ($p = 0.008$) was observed in the ipsilateral striatum. $[^{18}F]$FM-PBR28 PET studies exhibited an early peak uptake ratio of the ipsilateral and contralateral striatum than that of $[^{11}C]$PBR28 (3.9 at 35 min versus 4.3 at 115 min). The displacement PET study using unlabeled PBR28 30 min after the injection of $[^{18}F]$FM-PBR28 resulted in PET ligand washout, indicating the specific TSPO binding of $[^{18}F]$FM-PBR28 ($p = 0.029$). Co-injection with unlabeled FM-PBR28 effectively inhibited the uptake of $[^{18}F]$FM-PBR28 in the ipsilateral striatum ($p = 0.036$), but not with unlabeled flumazenil ($p = 0.250$), which is known to bind to the central benzodiazepine receptor, demonstrating $[^{18}F]$FM-PBR28 selectivity and specificity to the TSPO.

**Conclusion:** This study demonstrated that $[^{18}F]$FM-PBR28 is a promising TSPO PET ligand, using a novel direct radiofluorination method, for the introduction of the $[^{18}F]$fluoromethyl moiety. In addition, $[^{18}F]$FM-PBR28 exhibited an excellent TSPO specific ratio at early times compared with that of $[^{11}C]$PBR28. Thus, $[^{18}F]$FM-PBR28 is a specific TSPO PET ligand with a long half-life that provides an appropriate tool for the evaluation of neuroinflammatory disease.

**Key words:** $[^{18}F]$fluoromethyl PBR28, $[^{11}C]$PBR28, PET, Translocator protein, Neuroinflammation, Activated microglia

**Student Number:** 2007-30540
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Introduction

The activation of microglia is known as the main cellular response to neuroinflammation at the site of the central nervous system (1, 2). Activated microglia aggravate neuronal damage by releasing a several neurotoxins including interleukins, tumor necrosis factor-α, free radical, nitric oxide, proteinase and eicosanoids (3-8). In addition, the expression of a translocator protein (18 kDa; TSPO), which was formerly named the peripheral benzodiazepine receptor (PBR), is a reliable hallmark of microglial activation in neuroinflammatory diseases (9, 10). In fact, several studies have shown that increased TSPO density is an indication of neuronal damage or loss under neuroinflammatory disease, such as Alzheimer’s disease, Huntington’s disease, multiple sclerosis and ischemic brain injury (11-14). Therefore, successful development of a TSPO positron emission tomography (PET) ligand supplies clinical demand to monitor progression of neuroinflammation or measure therapeutic efficacy.

Currently, isoquinoline carboxamide derivative R-[N-methyl-\(^{11}\)C]PK11195,
as one of the first developed TSPO PET ligand, has been used in most of the PET imaging for neuroinflammatory studies over the past few decades (15). However, $[^{11}\text{C}]$PK11195 has several limitations, such as a high level of nonspecific binding and a low signal-to-noise ratio (16, 17). In fact, a previous study using a nonhuman primate brain reported that approximately 50% of the $[^{11}\text{C}]$PK11195 uptake was nonspecific (17). Therefore, a number of alternative TSPO PET ligands for neuroinflammatory imaging have been developed over the last several years (18).

Recently, $[^{11}\text{C}]$PBR28, a close analogue of DAA1106 (19), was developed for the imaging of neuroinflammation. $[^{11}\text{C}]$PBR28 exhibited a high specific signal to TSPO greater than that of $[^{11}\text{C}]$PK11195 in the nonhuman primate brain study and had adequate sensitivity to localize and quantify the expression of TSPO in a neuroinflammatory rat model (14, 20). However, the use of carbon-11 labeling in $[^{11}\text{C}]$PBR28 limits the clinical use of $[^{11}\text{C}]$PBR28 because of the short half-life of carbon-11 ($\beta^+; 99.8\%, T_{1/2} = 20.38\text{ min}$). In general, fluorine-18 has advantages over carbon-11 if a dynamic PET experiment has a turnover time longer than 100 min. $^{18}\text{F}$-labeled ligand also has a low positron energy (650 keV versus 960 keV) and
produce higher quality images with a higher spatial resolution in PET measurements.

In addition, fluorine-18 is convenient for long-term storage and long-distance transportation to other facilities. We thus designed a new TSPO PET ligand as a derivative of PBR28 containing a $[^{18}\text{F}]$fluoromethyl moiety (-CH$_2[^{18}\text{F}]$F)(Fig. 1). The $[^{18}\text{F}]$fluoromethyl moiety on PBR28 is sterically almost identical to -[^{11}\text{C}]CH$_3$, which exhibited promising results for TSPO imaging by PET, indicating that the biological properties of[^{11}\text{C}]PBR28 could be maintained when using the $[^{18}\text{F}]$fluoromethyl moiety on PBR28. The goals of this study were to detail the synthesis and radiofluorination of a newly developed TSPO PET ligand, $[^{18}\text{F}]$fluoromethyl PBR28 ($[^{18}\text{F}]$FM-PBR28, N-(2-$[^{18}\text{F}]$fluoromethoxybenzyl)-N-(4-phenoxyopyridin-3-yl)acetamide), and to evaluate the suitability of $[^{18}\text{F}]$FM-PBR28 as an indirect hallmark of neuroinflammatory disease.
Materials and Methods

General

All commercial reagents and solvents were used without further purification, unless otherwise specified. Reagents and solvents were commercially purchased from Sigma-Aldrich (St. Louis, MO, U.S.). \( \text{H}_2^{18}\text{O} \) was purchased from Taiyo Nippon Sanso Corporation (Tokyo, Japan). Fluorine-18 was produced at the Seoul National University Bundang Hospital by \( ^{18}\text{O}(p,n)^{18}\text{F} \) reaction through proton irradiation using a KIRAMS-13 cyclotron (KIRAMS, Seoul, Korea). The automated production of \([^{11}\text{C}] \text{PBR28} \) was performed in a TracerLab FX C pro (GE Healthcare, Milwaukee, WI, U.S.). tC18 Sep-Pak® cartridges were purchased from Waters Corp. (Milford, MA, U.S.). High-performance liquid chromatography (HPLC) purification was performed in a Gilson 322 (Waters, semi-Preparative Xterra RP-18, 10 µm, 10 x 250 mm) equipped with a NaI radiodetector (Raytest GmbH, Straubinghardt, Germany) and a UV-detector. HPLC-grade solvents (J. T. Baker Phillipsburg, NJ, USA) were used for HPLC purification after membrane filtering.
Radio-thin-layer-chromatography (radio-TLC) was analyzed on a Bioscan radio-TLC scanner (Washington DC, U.S.). All radioactivities were measured using a VDC-505 activity calibrator from Veenstra Instruments (Joure, Netherlands).


The novel radiotracer, [18F]FM-PBR28 was prepared by nucleophilic aliphatic substitution on the triazolium triflate-PBR28 precursor with fluorine-18 in a single-step radiolabeling procedure (Fig 2). Briefly, fluorine-18 was prepared by the $^{18}$O(p,n)$^{18}$F reaction using $\text{H}_2^{18}$O as the target material. $[^{18}\text{F}]^{\text{F}}/\text{H}_2^{18}$O was isolated from the enriched water by trapping in a Chromafix-HCO$_3$ cartridge (preactivated with 2 mL of ethanol and 5 mL of water) and then eluting with methanol:water (1:0.2 mL) dissolved 40% TBAHCO$_3$(1.7 μL). This solution was dried by azeotropic distillation with acetonitrile (0.3 mL) under nitrogen stream (x 2), and subsequently, triazolium triflate-PBR28 precursor (2.3 mg) in tert-butanol
(0.4 mL) was added. The reaction mixture was heated at 120 °C for 10 min. After the mixture was cooled to room temperature, the reaction mixture was diluted with 10 mL of water. This solution was loaded into a tC18 Sep-Pak cartridge, washed with 10 mL of water and eluted with 1.5 mL of CH$_3$CN. The combined solution was separated by a semi-prep HPLC system (Waters, Xterra RP-18, 10 x 250 mm, 10 μm) using a UV detector at 254 nm and a gamma-ray detector. Acetonitrile and water (45:55) were used as a mobile phase at a flow rate of 3 mL/min. The product fraction was collected after approximately 13.5 min. The fraction of $^{18}$F-FM-PBR28 collected from the HPLC system was diluted with 20 mL of water. The diluted solution was exchanged to 5% EtOH/saline solution by a tC18 Sep-Pak cartridge to remove the clinically unavailable HPLC solvent. For comparison, $^{[11]}$C- PBR28 was produced in an FX C pro module using $^{[11]}$C- MeOTf from the phenolic PBR28 precursor according to the literature with little modification (21-23).

2. *In vitro* TSPO binding affinity of PBR28 and FM-PBR28
Leukocytes were isolated from 50 mL heparinized whole blood by Ficoll-Hypaque density centrifugation using a Lymphocyte Separation Medium (Lonza, Walkersville, MD, U.S.) according to the manufacturer’s instructions. Following isolation, these leukocytes were viably cryopreserved. Prior to the day of the assay, the cells were thawed, diluted with an equal volume of buffer (50 mM HEPES, pH 7.4), homogenized with a Teflon pestle, and centrifuged at 20,000 x g for 15 min at 4 °C. The resulting crude membrane pellet was resuspended in 2.4 mL buffer and stored at -70 °C. The protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA, U.S.). For the binding assay, leukocytes (100 mL resuspended membranes) were added to the mixture containing 100 μL of radioligands ([³H]PK 11195 (S.A: 83.4 Ci/mmol), in 1x PBS) and 10% ethanol in a final volume of 1 mL for the saturation studies. For the inhibition studies, 1 mL of the reaction mixture containing 50 μL of FM-PBR28 or PBR28 (0.124-10,000 nM in 10% ethanol, n=4) and 0.07 nM radioligand ([³H]PK 11195) in 10% ethanol was incubated for 30 min at room temperature for the binding assay. The reaction mixture was filtered through Whatman GF/A glass filters and washed twice with 3
mL of 10% ethanol aliquots. The radioactivity retained on the filter was determined by a beta-counter. Under the assay conditions, the percent of the specific binding fraction was less than 20% of the total $^3$H radioactivity. The results of the inhibition and saturation experiments were subjected to non-linear regression analysis using PRISM software to calculate the half maximal inhibitory concentration (IC$_{50}$) values of FM-PBR28 and PBR28.

3. Lipophilicity of $[^{11}\text{C}]$PBR28 and $[^{18}\text{F}]$FM-PBR28

The log D value was measured four times by mixing a solution of $[^{18}\text{F}]$FM-PBR28 or $[^{11}\text{C}]$PBR28 (approximately 0.74 MBq) in 5% ethanol:saline (10 μL) with sodium phosphate buffer (0.15 M, pH 7.4, 5.0 mL) and n-octanol (5.0 mL) in a test tube. After vortexing for 1 min, each tube was then stored for 5 min at room temperature and the phases were separated. Samples of each phase (100 μL) were counted for radioactivity. Log D is expressed as the logarithm of the ratio of the counts per minute from n-octanol versus that of the sodium phosphate buffer.
4. Stability of $^{18}$F-FM-PBR28 in human serum and rat brain

4.1. In vitro stability in human serum

The stability of $^{18}$F-FM-PBR28 was assayed by monitoring the Radio-TLC profile and determining its radiochemical purity. To determine the in vitro serum stability, 100 μL of $^{18}$F-FM-PBR28 in 5% EtOH/saline was incubated with 0.5 mL of human serum at 37 °C for 4 h, and the solution was analyzed at 0, 10, 30, 60, 120 and 240 min by a radio-TLC scanner using MeOH-CH$_2$Cl$_2$ (10:90) as the developing solvent.

4.2. In vivo stability in rat brain and plasma (metabolism study)

$^{18}$F-FM-PBR28 (approximately 37 MBq, 0.5 mL of 5% EtOH:saline) was intravenously injected into the neuroinflammation rat models via a tail vein. After 30 and 60 min, the rat was sacrificed and samples of the brain were collected. The brain samples were homogenized in 2 mL of 50% CH$_3$CN-PBS (3 times) in a commercial blender for 3 min and centrifuged at 3,500 rpm for 5 min at 4 °C. The resulting supernatant was filtered through a 0.45 μm GH Polypro (GHP) membrane
Disc filter. The samples were analyzed with an authentic compound by HPLC as described above. The blood samples was centrifuged at 15,000 rpm for 1 min at 4 °C to separate the plasma (0.3 mL), which was collected in a test tube containing CH$_3$CN (0.5 mL) and a solution of the authentic FM-PBR (1.0 mg/2.0 mL of CH$_3$CN, 50 μL)(n=3). After the tube was vortexed for 15 s and centrifuged at 15,000 rpm for 2 min for deproteinization, the supernatant was collected. The resulting supernatant was filtered through a 0.45 μm GHP membrane Disc filter. The samples were analyzed by HPLC as described above.

5. PET imaging in an LPS-induced neuroinflammatory rat model

5.1. LPS-induced neuroinflammatory animal model

Male Sprague-Dawley rats weighing 200-250 g were used as the neuroinflammatory brain model using lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, U.S.). The rats were anesthetized with Ketamine HCL (50 mg/kg; Zoletil 50, Virbac, Carros, France) and Xylazine HCl (0.2 mg/kg; Rumpen, Bayer
Korea, Seoul, Korea) and placed on a stereotactic apparatus to immobilize the head. The skull was exposed, and a small hole was punctured with the use of a bone drill. Next, 50 μg of LPS was infused into the right striatum through the use of a Hamilton syringe at a flow rate of 0.5 mL/min (AP, 0.8 mm; L, -2.7 mm and P, -5.0 mm from the bregma)(24). The Hamilton syringe was sustained in place for 10 min to avoid backflow of LPS. The small hole in the skull was filled with wax, and the incised scalp was sutured (24). All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Seoul National University Bundang Hospital.

5.2. PET imaging protocol

Five rats (227.98 ± 13.8 g) were used for brain PET imaging at 4 days after LPS injection (25). Before the scan, the rats were anesthetized with 5% isoflurane and thereafter sustained with 1–2% isoflurane in a 7:3 mixture of N₂/O₂. PET imaging was performed in a dedicated small animal PET/CT (NanoPET/CT, Bioscan, Inc, Washington, DC, U.S.), with a 10 cm axial field of view (FOV) and a
12 cm transaxial FOV. This scanner yields a reconstructed PET spatial resolution of 1.2 mm full-width at half maximum at the center of the field of view. A CT scan of the head as a transmission map for attenuation correction was studied prior to the emission study. PET acquisition in the list mode was concomitantly started with the intravenous injection of either $^{11}$C-PBR28 or $^{18}$F-FM-PBR28 and was performed for 120 min. The first exam was studied with $^{11}$C-PBR28, and the second exam followed 3 h later with $^{18}$F-FM-PBR28.

At the end of each study, the list-mode data were sorted into a dynamic scan consisting of 64 frames. The acquired images were reconstructed by a 3-D Adjoint Monte Carlo method, combined with scatter and random corrections. Reconstructed voxel values in each frame are reported in units of kBq/cc, corrected for radioactive decay to the time of injection, and the voxel dimensions were 0.4 x 0.4 x 0.4 mm. The voxel values were then converted to units of percentage injected dose per milliliter of brain tissue per kilogram of body weight (%ID-kg/ml).

For the displacement PET study of $^{18}$F-FM-PBR28, unlabeled PBR28 (5 mg/kg intravenously) was administered 30 min after the injection of $^{18}$F-FM-
PBR28 (n=4; 226.9 ± 10.1 g)(26). The blocking PET study of $[^{18}\text{F}]$FM-PBR28 involved co-injection with unlabeled FM-PBR28 (5 mg/kg intravenously, n=3; 233 ± 6.2 g) and flumazenil (5 mg/kg intravenously, n=3; 220.9 ± 6.8 g).

5.3. Image analysis

Analysis of the PET images was performed using PMOD software v.3.1 (PMOD Technologies Ltd., Zurich, Switzerland). The volume of interest (VOI) with a 2 mm radius was delineated in the ipsilateral lesioned striatum by the intensely visualized region in the summed image of all the frames. The VOI of the lesion was copied and symmetrically plated into the contralateral striatum. The time activity curve (TAC) representing the variation in radioligand concentration according to the time course was estimated for the ipsilateral/contralateral striatum. The area under the time activity curve (AUC) was calculated to evaluate a single injection study of $[^{18}\text{F}]$FM-PBR28 or $[^{11}\text{C}]$PBR28. And, linear regression slope and specific binding ratio (SBR = (ipsilateral striatum - contralateral striatum)/contralateral striatum) were calculated for comparing two groups.
5.4. Immunohistochemical study

Immunohistochemical staining was performed on the neuroinflammatory rats just after the end of the PET study using $[^{18}F]FM$-PBR28. The rats were transcardially perfused under pentobarbital anesthesia (50 mg/kg, intraperitoneally) with 5 ml of phosphate buffered saline (PBS) followed by 20 mL of 4% paraformaldehyde (pH 7.4). The brains were quickly removed, post-fixed in the same fixative overnight at 4 °C, and then embedded in 30% sucrose solution for the following 3 days. Next, the brains were slowly frozen and maintained at -20 °C and sliced at 40 μm at the coronal section of the LPS-lesioned striatum. The sections were directly mounted onto slides and blocked with 4.5% normal goat serum and 0.1% triton 100 in PBS [60 min, room temperature (RT)]. Next, the slides were incubated with the primary antibody, mouse anti-CD68 (1:100; Serotec, Oxford, UK), overnight at 4 °C, and detected using the HRP-conjugated goat anti-mouse IgG antibody (1:500; Vector Laboratories, Burlingame, CA). Antibody reactivity was visualized using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame,
CA) and the diaminobenzidine (DAB) Zytomed kit (DAB057; Zytomed Systems, Berlin, Germany). After staining, a fluorescence microscope (Axio Observer, Zeiss, Germany) with Image-Pro Plus 7.0 software was used to histologically quantify the density ratio of CD68-positive activated microglia in the ipsilateral and contralateral striatum.

6. Statistical analysis

All of quantitative data are expressed as the mean ± SD. All comparisons were performed using Mann–Whitney nonparametric test. The statistical significance of the $p$ values was defined as $< 0.05$. The statistical analysis was performed using SPSS 12 (SPSS, Inc., Chicago, IL, USA).
Figure 1. The structure of $[^{11}\text{C}]$PBR28 and $[^{18}\text{F}]$FM-PBR28.

Figure 2. Radiosynthesis of $[^{18}\text{F}]$FM-PBR28.
RESULTS

1. Synthesis of radioligands

1.1. \[^{18}\text{F}\]FM-PBR28

\[^{18}\text{F}\]FM-PBR28 was prepared from nucleophilic aliphatic substitution on the triazolium triflate precursor of the PBR28 precursor with fluorine-18 in a single-step radiolabeling procedure. Among the various investigated conditions for fluorine-18 labeling, aprotic polar solvents, such as acetonitrile or DMF, which are well known as suitable \(S_{N2}\) reaction media, are not efficient for fluorine-18 incorporation of \[^{18}\text{F}\]FM-PBR28, resulting in less than 1% of radiochemical yield. This low yield is irrelevant compared to the increase of the precursor amounts or the reaction temperature from 80 to 150 °C. The likely cause of this low yield is that the precursor is decomposed because of the considerable basicity of the fluoride anion. A recent finding demonstrated that nucleophilic aliphatic fluorination via the \(S_{N2}\) mechanism is dramatically accelerated in tertiary alcohol solvents compared to that of aprotic polar solvents. Using this method, the labeling yield of \[^{18}\text{F}\]FM-PBR28
exhibited approximately 55% of radiolabeling yield when performed in tert-butanol as a reaction solvent at a reaction temperature of 120 °C for 10 min. While a 10 min reaction time was usually sufficient, the reaction time was extended to 15 min, as occasionally the radiochemical yield was slightly decreased. The increase of the precursor amounts or the change of the reaction solvent, such as tert-butanol:CH₃CN (8:2), resulted in a relatively lower yield of approximately 45%, and purification of [¹⁸F]FM-PBR28 from the reaction mixtures was also often difficult due to the many impurities, resulting in low specific activity. From the optimized condition described above, the radiochemical yield of the final formulated product, [¹⁸F]FM-PBR28, was 25.8 ± 3.2% (n=11, decay corrected) within a synthesis time of 55 min, including HPLC purification of the reaction mixture (Fig. 3). Specific activities (at the end of the synthesis) ranged from 279 ± 60 GBq/µmol, and the radiochemical purity of [¹⁸F]FM-PBR28 was over 99%. Co-injection of the radioactive product with an authentic standard of FM-PBR28 under different conditions (solvents, analytical HPLC columns (YMC-pro triart, 4.6 x 250 mm)) further established the identity of [¹⁸F]FM-PBR28 (Fig. 4). The formulated
radiotracer displayed no radiolysis for at least 120 min post formulation and required no stabilizing agents, such as ascorbate.

1.2. $^{[11]}C$PBR28 production

As previously reported, $^{[11]}C$PBR28 was efficiently and rapidly synthesized from its normethyl precursor (PBR-OH) and $^{[11]}C$CH$_3$OTf in an FX C-pro module (Fig. 5). The isolated radiochemical yield was $27.1 \pm 4.9\%$ (n=14, decay corrected), to yield approximately 100-120 mCi per batch, with $277 \pm 101$ GBq/μmol of specific activity at the end of the synthesis.

2. Receptor binding and lipophilicity of PBR28 and FM-PBR28

The affinity of FM-PBR28 and PBR28 for TSPO was measured by competition with $[^{3}]H$PK11195 in a membrane of human leukocytes for comparison purposes. In this assay, the fluoromethyl analogue, FM-PBR28, displayed a similar binding affinity to PBR28 ($IC_{50}$ values: $8.28 \pm 1.79$ versus $8.07 \pm 1.40$), suggesting
that substitution with an OCH$_3$F group did not obviously affect the affinity. This lack of effect may be due to the molecular similarity and bioisoteric property of the -OCH$_2$F and -OCH$_2$H groups. The octanol-buffer partition coefficient measurement resulted in a log D of 2.85 ± 0.02, which is similar to that of PBR28 (3.01 ± 0.01), as would have been predicted from the extension of the methoxy-substituent to a slightly larger fluoromethoxy-substituent (20).


3.1. In vitro stability in human serum

The results of [$^{18}$F]FM-PBR28 analysis in human serum demonstrated that the intact [$^{18}$F]FM-PBR28 has a stability of 98.8% for up to 240 min. This stability indicated that [$^{18}$F]FM-PBR28 was sufficiently stable to be used in further biological studies.

3.2. In vivo stability in rat brain and plasma
Figure 6 shows the percentages of unchanged $[^{18}\text{F}]$FM-PBR28 in the brain homogenate of rat measured by HPLC. The extraction ratios from the brain homogenate after 30 min and 60 min post-injection into the rat were approximately 92% and 89%, respectively. The amounts of $[^{18}\text{F}]$FM-PBR28 in the brain were 97.3% at 30 min and 96.8% at 60 min post-injection. No other radioactive metabolite was observed in the HPLC, except for approximately 2-3% of fluorine-18, at all time points examined. The percentage of radioactivity in rat brain extracts derived from the $[^{18}\text{F}]$FM-PBR28 metabolism was lower than that for $[^{11}\text{C}]$PBR28, which was compared with previously reported studies (27). The HPLC analysis of $[^{11}\text{C}]$PBR28 in the rat brain resulted in approximately 10-15% of the radioactive metabolite.

The extraction efficiency of the radioactivity into the CH$_3$CN supernatant was 78.5 ± 5.9% of the total radioactivity in the plasma. $[^{18}\text{F}]$FM-PBR28 in the plasma was rapidly broken down in the first 5 min post-injection and slowly declined for the remainder of the study as shown in Table 1. Metabolite-1 was the only radioactive metabolite in the plasma after 15 min post-injection though a little radioactive metabolite-2 exhibited in the plasma until 5 min post-injection.
4. PET imaging in the LPS-induced neuroinflammatory rat model

4.1. Neuroinflammatory PET study

The TACs in VOIs emerged for \[^{18}F\]FM-PBR28 and \[^{11}C\]PBR28 from the ipsilateral and contralateral striatum of the neuroinflammatory rat model (Fig. 7). The uptake of \[^{11}C\]PBR28 was slowly accumulated nearly 20 min after injection. After peaking, the uptake in the ipsilateral striatum tended to be stable state, whereas the uptake in the contralateral striatum slowly decreased until the end of the 120 min image after injection. Therefore, the highest uptake ratio of the ipsilateral to contralateral striatum appeared in the last scan (4.3 ± 0.7 at 115 min).

In addition, a significant difference between the ipsilateral and contralateral striatum curves was observed after injection of \[^{11}C\]PBR28 (AUC\(_{0-120\ \text{min}}\) = 46815.2 ± 12707.0 versus 14202.0 ± 2813.2, \(p = 0.008\)).

For \[^{18}F\]FM-PBR28, the uptake feature of the striatum was different from that of \[^{11}C\]PBR28. The uptake of \[^{18}F\]FM-PBR28 in the ipsilateral striatum rapidly
peaked in the first 4.5 min after injection and slowly declined in the remainder of the scan. However, the uptake in the contralateral striatum rapidly decreased during 30 min after injection, followed by a relatively slow decrease in the remainder of the scan. Therefore, the peak uptake ratio of the ipsilateral and contralateral striatum occurred at approximately 35 min after injection (3.9 ± 0.4). In addition, a significant difference existed between the ipsilateral and contralateral striatum curves (AUC_{0-120 min} = 42903.0 ± 8767.5 versus 12555.9 ± 2020.0, p = 0.008) after injection of [^{18}F]FM-PBR28. Comparing the uptakes of [^{18}F]FM-PBR28 and [^{11}C]PBR28, [^{18}F]FM-PBR28 PET studies exhibited an earlier peak uptake ratio of the ipsilateral and contralateral striatum (3.9 at 35 min versus 4.3 at 115 min) and a steeper decline after the peak than [^{11}C]PBR28 (-0.00120 ± 0.00051 versus -0.00042 ± 0.00023, p = 0.016).

4.2. Blocking study

The displacement study of [^{18}F]FM-PBR28 was performed by injecting an excess of unlabeled PBR28 (5 mg/kg) 30 min after the injection of [^{18}F]FM-PBR28.
when the uptake ratio of the ipsilateral and contralateral striatum reached a plateau at that time (Fig. 8). The uptake in the ipsilateral striatum after the injection of unlabeled PBR28 was rapidly displaced to a similar level with the uptake in the contralateral striatum. The SBR\textsubscript{50-70 min} after the injection of unlabeled PBR28 was significantly reduced with the SBR\textsubscript{10-30 min} before the injection of unlabeled PBR28 (0.4 ± 0.1 versus 2.4 ± 0.4, \( p = 0.029 \))(Fig. 9).

We also performed a \[^{18}\text{F}]\text{FM-PBR28}\) PET study with co-injection of unlabeled FM-PBR28 (5 mg/kg) and flumazenil (5 mg/kg). Compared with the baseline study of \[^{18}\text{F}]\text{FM-PBR28}\), the uptake of \[^{18}\text{F}]\text{FM-PBR28}\) in the ipsilateral striatum was not apparent after co-injection with unlabeled FM-PBR28 (Fig. 10). Actually, the co-injection of unlabeled FM-PBR28 resulted in a markedly decrease in \[^{18}\text{F}]\text{FM-PBR28}\) binding in the ipsilateral striatum compared with the baseline study of \[^{18}\text{F}]\text{FM-PBR28}\) (SBR\textsubscript{30-50 min} = 0.5 ± 0.2 versus 2.4 ± 0.7, \( p = 0.036 \))(Fig. 11). However, the co-injection with unlabeled flumazenil, which binds to the central benzodiazepine receptor, did not affect the uptake of \[^{18}\text{F}]\text{FM-PBR28}\) in the ipsilateral striatum, resulting in a similar uptake with that of the baseline study.
(SBR \textsubscript{30-50 min} = 2.4 \pm 0.7 versus 2.6 \pm 0.1, p = 0.250).

4.3. Immunohistochemistry study

In the neuroinflammatory rat model, a high density of CD68-positive activated microglia was observed in the ipsilateral striatum at 4 days after the LPS injection (Fig. 12A). However, the contralateral striatum exhibited a low density of CD68-positive activated microglia (Fig. 12B). In fact, the ipsilateral striatum of the neuroinflammatory rat model exhibited a 492.2 \pm 259.7 times higher density of CD68-positive activated microglia than that of the contralateral striatum.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>[\textsuperscript{18}F]FM-PBR28 (Parent)(%)</th>
<th>Metabolite-1 (%)</th>
<th>Metabolite-2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>69.0 \pm 4.7</td>
<td>27.9 \pm 5.2</td>
<td>3.1 \pm 1.1</td>
</tr>
<tr>
<td>5 min</td>
<td>26.8 \pm 4.1</td>
<td>67.9 \pm 5.1</td>
<td>5.2 \pm 1.2</td>
</tr>
<tr>
<td>15 min</td>
<td>24.6 \pm 0.6</td>
<td>75.4 \pm 0.6</td>
<td>0</td>
</tr>
<tr>
<td>60 min</td>
<td>20.8 \pm 20.8</td>
<td>79.2 \pm 0.6</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Analysis of [\textsuperscript{18}F]FM-PBR28 and its metabolites in rat plasma after intravenous injection of [\textsuperscript{18}F]FM-PBR28.
Figure 3. HPLC chromatogram of the reaction mixture (Xterra RP-18 (Waters, 10 x 250 mm) with a guard column (10 x 10 mm); eluant: 45% CH$_3$CN/H$_2$O; flow rate: 3 mL/min (blue line: UV-254 nm; red line: gamma-ray)).

Figure 4. HPLC co-injection chromatogram of pure [$^{18}$F]FM-PBR28 with an authentic compound (YMC-pro Triart (YMC, 4.6 x 250 mm); eluant: 45% CH$_3$CN/H$_2$O; flow rate: 1 mL/min (blue line: UV-254 nm; red line: gamma-ray)).
Figure 5. HPLC chromatogram of the reaction mixture (Xterra RP-18 (Waters, 10 x 250 mm) with a guard column (10 x 10 mm); eluant: 45% CH₃CN/H₂O; flow rate: 3 mL/min (upper: UV-254 nm; bottom: gamma-ray)).

Figure 6. HPLC in the brain homogenate after intravenous injection of [¹⁸F]FM-PBR28 to rat. HPLC condition: Xterra RP-18 (Waters); 7.9 x 250 mm; eluant: 40%
CH$_3$CN/H$_2$O; flow rate: 3 mL/min (upper: 30 min post-injection; bottom: 60 min post-injection; red line: gamma-ray; black line: UV-254 nm with authentic compound).

Figure 7. Time–activity curves and brain PET images of $[^{11}C]$PBR28 and $[^{18}F]$FM-PBR28 and a comparison between these radioligands. The Brain PET images are the summed images between 30 and 50 min after injection of the selected transaxial brain slice of the $[^{11}C]$PBR28 (D) and $[^{18}F]$FM-PBR28 (E)(each group, n=5).
Figure 8. Time–activity curves and brain PET images of $^{[18}F]FM$-PBR28 in the displacement study. The injection of unlabeled PBR 28 (5 mg/kg) was done at 30 min. The images shown at the right are the summed images taken before (B, 10-30 min) and after (C, 50-70 min) injection of PBR28 (n=4).

Figure 9. Specific binding ratio of $^{[18}F]FM$-PBR28 in the displacement study. The specific binding ratio before (10-30 min) and after (50-70 summed images) injection of PBR28 are shown (n=4).
Figure 10. $[^{18}F]$FM-PBR28 brain PET imaging of the neuroinflammatory rat model. The 30-50 min summed images of the selected transaxial brain slice of the baseline study (A) and the co-injection study of FM-PBR28 (B, 10 mg/kg) and flumazenil (C, 5 mg/kg) are shown.

Figure 11. Specific binding ratio of the neuroinflammatory rat model. The specific binding ratio of the baseline study and the co-injection study of FM-PBR28 (10 mg/kg) and flumazenil (5 mg/kg) are shown.
Discussion

We evaluated the ability of the newly developed \([^{18}\text{F}]\text{FM-PBR28}\) based on aryloxyanilide structures as a PBR radiotracer using a small animal PET scanner in the neuroinflammatory rat model with an LPS-lesioned ipsilateral striatum. A high uptake of \([^{18}\text{F}]\text{FM-PBR28}\) occurred in the ipsilateral striatum compared to that of the contralateral striatum in the neuroinflammatory rat model, resulting in similar uptake with that of the well-known TSPO radioligand, \([^{11}\text{C}]\text{PBR28}\). Furthermore, the high uptake of \([^{18}\text{F}]\text{FM-PBR28}\) in the ipsilateral striatum was consistent with the LPS-lesioned neuroinflammatory rat model with an upregulated TSPO density by activation of microglia (28-30). The uptake of \([^{18}\text{F}]\text{FM-PBR28}\) in the ipsilateral striatum was well-matched with the LPS-lesioned site using immunohistochemistry (Fig. 13). The ipsilateral striatum with intense \([^{18}\text{F}]\text{FM-PBR28}\) uptake (Fig. 13A: red arrow) exhibited a higher density of CD68-positive activated microglia (Fig. 13B: red square). However, the contralateral striatum exhibited no obvious uptake of \([^{18}\text{F}]\text{FM-PBR28}\) (Fig. 13A: blue arrow) and a low density of CD68-positive
activated microglia (Fig. 13B: blue square). Overall, \([^{18}F]FM-PBR28\) was observed to enter the neuroinflammatory rat brain by crossing the blood-brain barrier and specifically binding to the high TSPO density lesion of the ipsilateral striatum.

After peaking, \([^{18}F]FM-PBR28\) exhibited a steeper decline in the remainder of the scan than \([^{11}C]PBR28\) \((p = 0.016)\). The reason for this different tendency is unclear, but it could be related with earlier reduced brain reentry of \([^{18}F]FM-PBR28\) than \([^{11}C]PBR28\) \((26.8\% \text{ versus } 53.5\% \text{ of remnant PET ligand in rat plasma at 5 min, respectively})\)(27). The chemical feature of fluoromethyl group with demethylation, which is resulted in defluorination and skull-bound radioactivity, might be another cause of the rapidly decline slope of \([^{18}F]FM-PBR28\) (31-32).

The displacement study of \([^{18}F]FM-PBR28\) revealed that the uptake in the ipsilateral striatum rapidly decreased and reached the level of uptake in the contralateral striatum after injection of unlabeled PBR28. And, we could roughly estimate the specific binding by comparing the uptake between baseline study of \([^{18}F]FM-PBR28\) and co-injection study with unlabeled FM-PBR28. Using this
estimation approach, co-injection with unlabeled FM-PBR28 decreased 77.4% of the uptake of $^{18}$F-FM-PBR28. In comparison with our co-injection study with FM-PBR28, co-injection with unlabeled flumazenil, which binds to the central benzodiazepine receptor, did not interfere the uptake of $^{18}$F-FM-PBR28 (-0.2%).

There are several differences between the neuroinflammatory PET images of $^{18}$F-FM-PBR28 and $^{11}$C-PBR28. $^{18}$F-FM-PBR28 reached the peak uptake ratio of the ipsilateral and contralateral striatum after injection more quickly than that of $^{11}$C-PBR28 (3.9 at 35 min versus 4.3 at 115 min). In contrast to $^{11}$C-PBR28, $^{18}$F-FM-PBR28 is convenient for long-term storage and can be widely used in the clinic or hospital without the installation of a cyclotron (109.74 min versus 20.38 min).
Figure 12. The Immunohistochemical staining (anti-CD68; Serotec, Oxford, UK) of the ipsilateral (A: 20X) and contralateral striatum (B: 20X) in the neuroinflammatory rat model.

Figure 13. The 30-50 min summed brain PET image using $[^{18}\text{F}]\text{FM-PBR28}$ (A) and the image of the immunohistochemically stained rat brain (B; anti-CD68; Serotec, Oxford, UK) of the neuroinflammatory rat model (coronal view).
Conclusion

We successfully developed a promising TSPO PET radioligand, $^{[18}F]FM$-PBR28, for the PET imaging of neuroinflammation that uses a novel direct radiofluorination method for the introduction of the $^{[18}F]$fluoromethyl moiety. $^{[18}F]FM$-PBR28 can be used to replace carbon-11 and exhibits an excellent TSPO specific ratio at early times compared with $^{[11}C]PBR28$. Therefore, we can apply $^{[18}F]FM$-PBR28 as an appropriate tool for the evaluation of neuroinflammatory disease.
References


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국문 초록

TSPO PET 방사성추적자

$[^{18}\text{F}]$Fluoromethyl PBR28을 이용한 신경염증영상 연구

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김 범 산

목적: 중추 신경계 질환에서 미세아교세포의 translocator protein (18 kDa)(TSPO)의 발현은 신경 염증 과정 중의 세포 활성화를 평가하는 생체 내 바이오 마커로 활용할 수 있다. 본 연구는 새로운 TSPO PET 방사성추적자로 $^{18}\text{F}$ 표지 fluoromethyl moiety를 가진 PBR28 유도체($[^{18}\text{F}]\text{FM-PBR28}$)의 합성 및 신경 염증 질환에서 $[^{18}\text{F}]\text{FM-PBR28}$의 유용성을 평가하고자 한다.
방법: 

[^{18}F]FM-PBR28은 PBR28-OH에 triazolium triflate를 도입한 화합물을 전구체로 사용하고 일반적인 과정으로 ^{18}F를 치환하는 새로운 방법으로 합성하였다. 

[^{11}C]PBR28은 기존의 알려진 방법에 따라 phenolic PBR28 전구체에[^{11}C]MeOTf를 이용, FX C pro module에서 합성하였다. FM-PBR28과 PBR28의 TSPO에 대한 체외 affinity(IC_{50})는 사람 백혈구 막에서[^{3}H]PK11195와의 경쟁적 결합으로 그리고 lipophilicity는 octanol-buffer 분배 계수 (partition coefficient)로 측정하였다. 

[^{18}F]FM-PBR28 및[^{11}C]PBR28 PET study는 lipopolysaccharide (LPS)를 편측 신조체에 주입 후 4일 된 신경 염증 쥐 모델에서 시행하였다 (each group, n=5). 또한,[^{18}F]FM-PBR28의 특이도 (specificity)와 선택성 (selectivity)을 평가하고자 PBR28 (5 mg/kg)를 사용하여 displacement PET study (n=4)를 하였고, FM-PBR28 (5 mg/kg) 그리고 flumazenil (5 mg/kg)를 사용하여 co-injection PET study (each group, n=3)를 시행하였다. 

결과: 

[^{18}F]FM-PBR28의 방사화학적 수율은 25.8 ± 3.2%이었으며, 방사화학적 순도는 99% 이상이었다. [^{18}F]FM-PBR28은 reverse-phase HPLC system으로 분리/정제하였으며, tC18 Sep-Pak cartridge를 이용, 5% EtOH/saline으로 제조하였다. 

[^{11}C]CO_{2}를 이용한[^{11}C]PBR28의 방사화학적 수율은 27.1 ± 4.9%이었다. FM-PBR28과 PBR28의 체외 affinity (IC_{50})와 log D는 서로 유사한
함을 확인하였다 (IC₅₀: 8.28 versus 8.07 nM; Log D: 2.85 versus 3.01).

신경염증 모델 쥐에서 촬영한 [¹⁸F]FM-PBR28 PET study에서 LPS를 주사한 편측 선조체는 반대측 선조체에 비하여 3.4배 이상의 높은 섭취를 보였으며 (p = 0.008), [¹¹C]PBR28 영상과 비교하였을 때 조기에 높은 좌우 선조체 비를 보였다 (3.9 at 35 분 versus 4.3 at 115 분). Displacement PET study에서 [¹⁸F]FM-PBR28 주사 30분 후에 PBR28의 주입은 편측 선조체의 급격한 섭취감소를 보임으로써 [¹⁸F]FM-PBR28의 특이적인 TSPO binding을 확인할 수 있었다 (p = 0.029). Co-injection PET study에서 [¹⁸F]FM-PBR28과 동시에 FM-PBR28를 주입하였을 때, 편측 선조체의 섭취가 효과적으로 저하됨을 확인하였다 (p = 0.036). 그러나 central benzodiazepine receptor에 특이적으로 결합하는 flumazenil의 동시주입은 편측 선조체의 섭취에 영향을 주지 않으며 (p = 0.250), 이를 통하여 [¹⁸F]FM-PBR28은 TSPO에 선택적으로 결합한다는 것을 알 수 있었다.

중심단어: \[^{18}\text{F}]\text{fluoromethyl PBR28}, \[^{11}\text{C}]\text{PBR28}, \text{양전자단층촬영, Translocator protein, 신경염증, 활성 미세아교세포}

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