Radiosynthesis and Biodistribution in Mice of a $^{18}$F-Labeled Analog of O-1302 for Use in Cerebral CB1 Cannabinoid Receptor Imaging

Yumiko Nojiri, Kiichi Ishiwata, Qinggeletu, Shintaro Tobiishi, Toru Sasada, Fumihiko Yamamoto, Takahiro Mukai, and Minoru Maeda

*Graduate School of Pharmaceutical Sciences, Kyushu University; 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan; and Positron Medical Center, Tokyo Metropolitan Institute of Gerontology, 1–1 Naka-cho, Itabashi-ku, Tokyo 173–0022, Japan.

The suitability of an $^{18}$F-labeled form of $N$- (piperidin-1-yl)-1-(2,4-dichlorophenyl)-5- {4′- (5-fluoropentyl)-phenoxy}-4-methyl-1H-pyrazole-3-carboxamide (I), a CB1 cannabinoid ligand with high binding affinity ($K_i$ = 0.91 nM) and moderate lipophilicity ($\log P_{12}$ = 2.9), as a radiotracer for positron emission tomography imaging was evaluated in mice. Ligand I was labeled with $^{18}$F ($T_{1/2}$ = 109.7 min) by treatment of the corresponding tosyl derivative with $[^{18}$F]fluoride ion in acetonitrile. Tissue distribution studies of the $^{18}$F-labeled form ($[^{18}$F]I) in mice demonstrated low brain uptake with minimal specific binding in brain regions.

Cyclosporin A (a modulator of P-glycoprotein) treatment significantly increased both the brain uptake and the brain-to-blood ratio of $[^{18}$F]I, indicating the possibility that P-glycoprotein regulates the ability of $[^{18}$F]I to cross the blood brain barrier. Radioligand $[^{18}$F]I does not have the required properties for imaging the cerebral cannabinoid CB1 receptor in \textit{vivo}.

Key words: CB1 cannabinoid ligand; brain distribution; fluorine-18; radiopharmaceutical

The cannabinoid CB1 receptor, the G-protein-coupled membrane receptor localized presynaptically, is the most abundant cannabinoid receptor subtype found in the central nervous system.\(^1\) The invention of SR141716 (also known as rimonabant) as the first high-affinity CB1-selective antagonist has led to the development of a large variety of cannabinoid CB1 ligands, characterized by a cyclic heteroaromatic moiety as their core structure, such as aminoalkylindoles and pyrazoles.\(^2\) The cerebral CB1 receptor ligands have therapeutic potential in a range of disorders including pain, weight gain, emesis and mood disorders.\(^5\)\(^6\) \textit{In vivo} imaging of CB1 regional brain distribution using positron emission tomography (PET) or single photon emission computed tomography (SPECT) may provide an important tool to study the roles of the cannabinoid CB1 receptor in a variety of disorders and for the development of cannabinergic medications. Most reported efforts at developing \textit{in vivo} imaging of the CB1 receptor have focused on radioligands with structural similarity to SR141716 which are highly lipophilic.\(^3\)\(^4\)\(^9\)\(^10\) Until recently, no derivatives structurally related to SR141716 have proven to be useful for \textit{in vivo} imaging when prepared as radioligands, due to poor brain entry, high levels of nonspecific binding, or low levels of specific binding in brain regions. Nevertheless, the challenge still exists to find suitable analogs based on a pyrazolyl nucleus, aiming at improvement of the imaging properties. Recent efforts have been devoted to designing CB1 ligands with lower lipophilicity so as to share favorable pharmacological activity \textit{in vivo}. Promising data have more recently been reported with \textit{[^{18}$C]JHU75528, bearing a pyrazole core ring, which was found to be a selective and effective radiotracer for imaging the CB1 receptor in rodents \textit{in vivo}.

In addition, researchers at Merck developed a high-affinity CB1 receptor ligand, \textit{$^{18}$F}-labeled MK-9470, which has an acyclic chiral unit, and which produced the first successful PET imaging of the brain CB1 receptor in a monkey and a human.\(^9\) We have recently developed \textit{N}- (piperidin-1-yl)-1-(2,4-
dichlorophenyl)-5- {4′- (5-fluoropentyl)phenox y}-4-methyl-1H-pyrazole-3-carboxamide (I) based on \textit{N}- (piperidin-1-yl)-1-(2,4-dichlorophenyl)-4-methyl-5- (4-pentylphenyl)-1H-pyrazole-3-carboxamide (O-1302),\(^19\) also a close structural analog of SR141716, as a CB1 cannabinoid ligand with good binding affinity ($K_i$ = 0.91 nM), while showing a lower lipophilicity (experimental log $P_{12}$ = 2.9) compared to SR141716 (experimental log $P_{12}$ = 3.8).\(^20\) This paper describes the radiosynthesis and evaluation of the potential of the \textit{18}F-labeled form of I ($[^{18}$F]I) in detecting the brain CB1 receptor \textit{in vivo}.

MATERIALS AND METHODS

$\textit{N}$- (Piperidin-1-yl)-1-(2,4-dichlorophenyl)-5- {4′- (5-fluoropentyl)phenox y}-4-methyl-1H-pyrazole-3-carboxamide (I) as an unlabeled standard, and \textit{N}- (piperidin-1-yl)-1-(2,4-dichlorophenyl)-4-methyl-5- {4′- (5-p-toluensulfonyloxy-pentyl)phenox y}-4-methyl-1H-pyrazole-3-carboxamide (2) as a precursor for labeling with fluorine-18, were synthesized according to already reported procedures.\(^20\) SR141716 was also prepared according to the published method.\(^3\) All chemicals and solvents were obtained from commercial sources. Male ddY mice (8 weeks old, 35–1.3 g) were obtained from Tokyo Laboratory Animals Co., Ltd. (Tokyo, Japan). The animals received a standard diet and water with free access. The animal studies were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology.

No-carrier-added aqueous $[^{18}$F]fluoride ion was produced on an AVF compact cyclotron (CYPRIS 370, 74 cm in diameter; Sumitomo Heavy Industries, Ltd., Tokyo, Japan) by 20-to-30 min irradiation of a 2 ml water target using 17 MeV proton beams on 95.7% enriched $[^{18}$O]H₂O (Osaka Oxygen Co., Osaka, Japan) by a $[^{18}$O(p,n)$[^{18}$F] reaction. To recover the $[^{18}$O]H₂O, the batch of aqueous $[^{18}$F]fluoride ion was passed through an anion exchange resin (BioRad AG-1 X8, 100—200 mesh, 1.6-mm inner diameter×20-mm length), precon-
abeled with 1 m K₂CO₃ (5 ml) and water (10 ml). The [¹⁸F]fluoride was eluted from the resin with a solution of K₂CO₃ (50 mmol/l, 0.4 ml) in water. The recovered [¹⁸F]fluoride was added to a solution of Kryptofix 2.2.2 (20 mg) in acetonitrile (1.2 ml) in a septum-sealed vial. The vial was heated at 130 °C, and the solvent evaporated to dryness under a stream of helium flow (50—100 ml/min) for 5—10 min. The residue was dissolved in dry acetonitrile (1 ml), and dried by azotropic evaporation three times.

N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-4-methyl-5-{4-[¹⁸F]fluorophenyl}-1H-pyrazole-3-carboxamide (I[¹⁸F]I) One milligram of precursor (2) dissolved in anhydrous acetonitrile (0.5 ml) was added to the reaction vial containing a Kryptofix 2.2.2—K[¹⁸F] complex and the mixture was heated at 120 °C for 30 min. The reaction mixture was then diluted with 0.4 ml of H₂O and 0.4 ml of acetonitrile/0.1% aqueous trifluoroacetic acid (TFA) (6/4, v/v), and applied to high-performance liquid chromatography (HPLC) using a reversed phase column (YMC-Pack Pro C18 RS, 10-μm inner diameter×100-mm length, Tosoh Co., Ltd., Tokyo, Japan), comprising a UV absorbance (260 nm) with an NaI(Tl) scintillation detector. The mobile phase was a mixture of acetonitrile/0.1% aqueous TFA (6/4, v/v) at a flow rate of 6 ml/min. The [¹⁸F]I fraction (retention time: 20.0—20.5 min) was collected in a flask containing 0.05 ml of 100 mg/ml ascorbate and evaporated to dryness. The residue was dissolved in physiological saline containing 0.125% Tween 80 for in vivo experiments. The isolated radiochemical yield at the end of synthesis was 7.4—11.1%. Radiochemical purity as assessed by analytical HPLC was >96% and the specific radioactivity was 22—58 MBq/nmol at the end of synthesis.

Analytical HPLC: column, TSKgel Super-ODS (4.6-mm inner diameter×100-mm length, Tosoh Co., Ltd., Tokyo, Japan); mobile phase, acetonitrile/50 mm acetic acid/50 mm ammonium acetate (7:1.5/1.5, v/v/v); flow rate, 1.0 ml/min; and retention time: 4.9—5.1 min for [¹⁸F]I.

Tissue Distribution of the Tracer in Mice Radioligand [¹⁸F]I (1.0 MBq, 0.056 nmol) was intravenously injected into mice. They were killed by cervical dislocation at 5, 15, 30, 60 and 120 min after injection (n=4). Blood was collected by heart puncture and the tissues were harvested. The [¹⁸F]I in the samples was counted with an auto-gamma-counter and the tissues were weighed. The tissue uptake of [¹⁸F]I was expressed as a percentage of injected dose per gram of tissue (%ID/g).

In another group of mice, [¹⁸F]I (1.0 MBq, 0.035 nmol) was injected 15 min after the intravenous administration of each of unabeled I and SR141716 (CB1-selective inverse agonist, Ki=1.3 nM). Each blocker was dissolved in dimethyl sulfoxide (DMSO) and the injected dose was 1 mg/kg. In the control mice the same amount of DMSO was injected. One-hundred and twenty minutes later the mice were killed (n=6—7), and blood and brain were harvested. The brain was divided into the cerebral cortex, hippocampus, striatum, hypothalamus, midbrain, cerebellum and medulla oblongata. The tissue uptake of [¹⁸F]I was expressed as a %ID/g. To investigate the effect of the P-glycoprotein (P-gp) inhibitor on the tracer uptake, the mice were treated with an intravenous injection of cyclosporin A (CysA) at 50 mg/kg. After 30 min of treatment, [¹⁸F]I (1.2 MBq, 0.12 nmol) in DMSO was intravenously injected into the mice. The radioactivity in blood and brain was measured at 120 min after injection of the tracer and the results were expressed as %ID/g. The concentration of CysA (50 mg/kg) used in this experiment has been reported to have a biochemical effect leading to inhibition of the efflux action of P-gp in the rodent brain.²²

Metabolite Analysis Radioligand [¹⁸F]I (8.0—9.8 MBq, 0.16—0.44 nmol) was intravenously injected into mice, and 120 min later they were killed by cervical dislocation (n=3). The blood was removed by heart puncture using a heparinized syringe, and the brain was removed. The blood was centrifuged at 7000×g for 1 min at 4 °C to obtain plasma, which was denatured with an equal equivalent volume of acetonitrile. The cerebral cortex (approximately 200 mg) was homogenized with an equal equivalent volume of acetonitrile.

Each of the mixture was centrifuged under the same conditions, and the supernatant was analyzed by thin-layer chromatography (TLC). Thirty microliters of each supernatant were spotted on silica gel plates (Kieselgel 60 plates, Merck Co., Tokyo, Japan). After developing with hexane:EtOAc (1:4), the plates were dried and apposed on a storage phosphor screen (Phosphor Imager SI System, Molecular Dynamics, Sunnyvale, CA, U.S.A.). The location of [¹⁸F]I on the plates was determined by co-spotting with an authentic sample.

Statistical Analysis Student’s t-test was performed to identify any significant differences between the control and the mice pretreated with blockers or CysA in the tissue distribution studies.

RESULTS AND DISCUSSION

The reaction of a sulfonate ester with [¹⁸F]fluoride is an excellent method for fluorine-18 labeling. ²³ N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-{4-[5-fluoro-pentyl]phenyl}-4-methyl-1H-pyrazole-3-carboxamide (I) was labeled with fluorine-18 from the corresponding tosylxy analog 2 using the one-step radiochemical process outlined in Fig. 1.

The nucleophilic substitution of tosylated precursor 2 with the cyclotron-produced [¹⁸F]fluoride as the no-carrier-added, activated K[¹⁸F]-Kryptofix 2.2.2 complex was performed in acetonitrile for 30 min at 120 °C. Substrate 2 was sensitive to bases and degraded with heating during fluorination and no efforts were made to identify side products. [¹⁸F]I could be

Fig. 1. Structures of SR141716, O-1302 and Fluorinated O-1302 (1) Cannabinoid Receptor Antagonist, and Radiosynthesis of [¹⁸F]I by Nucleophilic Displacement on a Tosylate Precursor (2)
obtained with >96% radiochemical purity with specific radioactivities ranging from 22 to 58 MBq/nmol after HPLC purification of the crude product, and was completely separated from non-radioactive precursor, as well as from non-radioactive unidentified side products, as verified by HPLC. The isolated radiochemical yield was 7.4—11.1% non-decay-corrected yield (based on starting $^{18}$F-fluoride) at the end of synthesis and was not optimized because the radiosynthesis provided $^{[18}$F$]I$ in sufficient quantities to conduct initial in vivo studies using animals, with a reasonable reproducibility. The total radiosynthetic time, including HPLC purification, took about 90 min.

After $^{[18}$F$]I$ was injected intravenously into mice, the biodistribution of the tracer was determined as a function of time after injection, as shown in Table 1. The highest tracer uptake (mean %ID/g) was observed in the liver (16.3), followed by the kidney (5.8), and heart (5.0) at the first sacrifice time point of 5 min post injection and these initial uptakes were followed by a relatively slow clearance during the observation period (2 h). Blood radioactivity was low at 5 min (3.9% ID/g) and fell to <1.5% ID/g within 30 min after injection. The bone radioactivity was initially low and then showed a gradual increase from 1.10% ID/g at 5 min to 2.98% ID/g at 120 min, indicative of relative stability toward in vivo metabolic defluorination. The initial uptake of $^{[18}$F$]I$ in the whole brain was low (0.26% ID/g) at 5 min post injection and the level slightly increased (0.30% ID/g) during the next 10 min, and thereafter slightly declined from 60 to 120 min post injection.

The stability of $^{[18}$F$]I$ was determined by silica gel TLC of radioactivity extracted from blood samples and brain tissue at 120 min after injection. Only 5.7±4.1% (mean±standard error) of the total plasma radioactivity was unchanged and the remaining plasma radioactivity was composed of three distinct metabolites which were more hydrophilic than $^{[18}$F$]I$. On the other hand, in the brain, $^{[18}$F$]I$ was relatively stable since 78.6±4.7% of the radioactivity in the brain homogenates was detected as an unchanged form, together with minor radioactive metabolites which were more hydrophilic than the parent radioligand. These results suggested that the radioligand itself was mostly responsible for the observed distribution of brain radioactivity.

The regional brain distribution of $^{[18}$F$]I$ was studied at the 120-min time point, because the radioactivity levels in the brain remained for 120 min. As shown in Table 2, $^{[18}$F$]I$ entering the brain was almost uniformly distributed in the different regions, and such obscured regional contrast of radioactivity distribution was also observed by ex vivo autoradiography using brain sections (data not shown), supporting the results of the dissection experiments. Thus, the observed distribution did not clearly reflect the expected one which is based on the known gross densities of the CB1 receptor in the brain of mammalian species (cerebellum> hippocampus> striatum> cerebral cortex> brainstem, thalamus). Although this observation was not encouraging enough to warrant a blocking study, we examined the effect of prior treatment with unlabeled I on the brain uptake of $^{[18}$F$]I$. As can be seen from Table 2, pre-administration of unlabeled I (1 mg/kg, 15 min prior to the tracer injection) did not significantly alter the tracer distribution. However, pre-treatment with a selective CB1 antagonist, SR141716 (1 mg/kg), using a dose which has been shown to be sufficient to block tracer accumulation in the mouse brain, significantly reduced the radioactivity levels of $^{[18}$F$]I$ to about 70% of the control in the cerebral cortex, midbrain and medulla oblongata. This reduction, defined as the displacable radioactivity, seemingly indicates that a small fraction of the total radioactivity in the brain belongs to the specific binding of the radioligand to the CB1 receptor in these regions. Usually, an unlabeled carrier may be more effective to block the specific binding of the radioligands, if the specific binding is present. In the present study the concentrations of unlabeled carrier I in the brain may be much lower than that of SR141716 after pre-treatment with the same dose (1 mg/kg), because $^{[18}$F$]I$ was pumped out from the brain by the function of P-gp, as described below. Consequently, it is conceivable that pre-treatment with unlabeled I resulted in a very small reduction in the regional brain uptake of $^{[18}$F$]I$ without statistical significance.

The adequate distribution of a radiotracer to the central nervous system is one of the key prerequisites for its effective use in the brain. A number of studies have shown that no single factor affects the brain uptake of a molecule from blood circulation, although lipophilicity seems to exhibit a strong influence on this process. The brain uptake of $^{[18}$F$]I$ at 5 min post injection showed low value (0.26% ID/g) and the

| Table 1. Tissue Distribution of Radioactivity of Mice after Intravenous Injection of $^{[18}$F$]I$
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<td>a) Mean±S.D. (n=4).</td>
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| Table 2. Effects of Carrier and SR141716 on Radioactivity Accumulation of $^{[18}$F$]I$ in Mouse Brain and Blood at 120 min after Intravenous Injection of Tracer
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<td>a) Mean±S.D. (n=6—7).</td>
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<td>b) Carrier (unlabeled I)</td>
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p<0.001 (Student’s t-test, compared with control).
blood level at this time point was relatively high, both of which are very unfavorable. The lipophilicity of I, somewhat lower than that of SR141716, is on a level generally desirable for prospective brain imaging (log \( P = 1 - 3 \)).\(^{26,27}\) but its brain accumulation was much lower than expected, thus demonstrating low initial permeability on crossing the blood brain barrier (BBB).

Many lipophilic compounds may often be substrates of the P-gp found in BBB, which promptly transport these compounds back into the blood.\(^{28}\) Recently, the modulation of several radioligands by P-gp in vivo has been evaluated in rodents, showing that P-gp plays an important role in limiting the distribution of radioligands to the brain.\(^{22,29}\) We examined whether the brain uptake of \([^{18}\text{F}]\text{I}\) was affected by treatment with CysA, a modulator of P-gp. As shown in Table 3, CysA treatment increased both the brain uptake and the brain-to-blood ratio, although at a single time point (120 min), indicating the contribution of P-gp-mediated transport in the brain distribution of \([^{18}\text{F}]\text{I}\). These findings offer a partial explanation for the low brain accumulation of \([^{18}\text{F}]\text{I}\) at tracer dose. The CysA treatment at the 50 mg/kg dose used in the present study greatly altered blood clearance of the radioligand, probably because the permeability of the radioligand into several tissues (including the brain) may be affected.\(^{21,22}\) This is one reason why two parameters (brain uptake and the brain-to-blood ratio) were evaluated (Table 3). In order to evaluate the modulation of P-gp for the radioligand, dynamic evaluation such as PET is much more preferable, however, the data at a single time point are reasonably useful for evaluation of the modulation of P-gp.\(^{22}\)

Most of the CB1 inhibitor pharmaceuticals used as a basis for developing CB1 receptor imaging radiotracers are highly lipophilic, which appear to be necessary for high-affinity binding to the active site region.\(^{30}\) It is considered that this often leads to high non-specific brain distribution of radioligands, even when the ligands have high binding affinity to this target, as pointed out by others. The lowest possible lipophilicity is generally desirable for minimizing the non-specific binding a radioligand.\(^{31}\) Currently, there is no sufficiently general understanding of the relationship between lipophilicity and the in vivo binding characteristics of radioligands investigated for imaging the CB1 receptor.\(^{14,32}\) Recently, there have been some attempts to prepare analogs which reduce lipophilicity more than existing CB1 ligands.\(^{33,34}\) In the present study we could not clearly prove that the brain accumulation of \([^{18}\text{F}]\text{I}\), with limited delivery to the CNS, was due to the binding of the ligand to CB1 receptor binding sites, although a small fraction of displacable binding was present in the mouse brain. This observation leads to the indication that the measured log \( P \) value of 2.9 for a pyrazolyl ligand such as \([^{18}\text{F}]\text{I}\) is not so lipophilic that it would have prohibitively non-specific binding. On the other hand, the possibility of the affinity of \([^{18}\text{F}]\text{I}\) to other binding sites besides the CB1 receptor can not be ruled out because we used only SR141716 as a blocker in the present study.

In conclusion, despite a high in vitro binding affinity and moderate lipophilicity of the new analog, \([^{18}\text{F}]\text{I}\) is not suitable for imaging the CB1 receptor in the brain because of poor brain entry and high levels of non-specific binding which masked the interaction with specific binding sites. The results of this work also demonstrate the poor ability to predict the in vivo behavior of a radiotracer molecule based on its log \( P \) value and affinity alone, thus emphasizing the need for much more knowledge in this area. Further effort will be focused on the design of a radiotracer based on alternative structural templates.

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