Development of an In Vivo Method to Estimate Effective Drug Doses and Quantify Fatty Acid Amide Hydrolase in Rodent Brain using Positron Emission Tomography Tracer [11C]DFMC

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ABSTRACT

Fatty acid amide hydrolase (FAAH) is a key enzyme in the endocannabinoid system. N-(3,4-Dimethylisoxazol-5-yl)piperazine-4-[4-(2-fluoro-4-[11C]methylphenyl)thiazol-2-yl]-1-carboxamide ([11C]DFMC) was developed as an irreversible-type positron emission tomography (PET) tracer for FAAH. Here, we attempted to noninvasively estimate rate constant k3 (rate of transfer to the specifically-bound compartment) as a direct index for FAAH in the rat brain. First, the two-tissue compartment model analysis including three parameters [K1, k0, and k2] (two-tissue compartment model for the irreversible-type radiotracer [2TCMi]) in PET study with [11C]DFMC was conducted, which provided 0.21 ml·cm−2·min−2 of the net uptake value (K), an indirect index for FAAH, in the FAHH-richer region (the cingulate cortex). Subsequently, to noninvasively estimate K1, the reference model analysis (Pattak graphical analysis reference model) was tried using a time-activity curve of the spinal cord. In that result, the noninvasive K1 value (KREF) was concisely estimated with high correlation (r > 0.95) to K1 values based on 2TCMi. Using estimated KREF value, we tried to obtain calculated k3 based on previously defined equations. The calculated k3 was successfully estimated with high correlation (r = 0.95) to direct k3 in 2TCMi. Finally, the dose relationship study using calculated k3 demonstrated that in vivo ED50 value of [3-(3-carbamoylphenyl)phenyl]N-cyclohexylcarbamate, a major inhibitor of FAAH, was 66.4 μg/kg in rat brain. In conclusion, we proposed the calculated k3 as an alternative index corresponding to regional FAAH concentrations and suggested that PET with [11C]DFMC enables occupancy study for new pharmaceuticals targeting FAAH.

SIGNIFICANCE STATEMENT

In the present study, we proposed calculated k3 as an alternative index corresponding with fatty acid amide hydrolase concentration. By using calculated k3, in vivo ED50 of [3-(3-carbamoylphenyl)phenyl]N-cyclohexylcarbamate was successfully estimated to be 66.4 μg/kg for rats. Thus, we demonstrated the pharmacological utility of positron emission tomography with N-(3,4-dimethylisoxazol-5-yl)piperazine-4-[4-(2-fluoro-4-[11C]methylphenyl)thiazol-2-yl]-1-carboxamide.

Introduction

The endocannabinoid system is known as a key biologic system having retrograde neurotransmission in the central nervous system (Devane et al., 1992; Bayewitch et al., 1995) and has been reported to regulate a broad range of physiologic processes in multiple disorders, such as pain, neuroinflammation, anxiety, neurodegenerative disorders, cancer, epilepsy, and metabolic syndrome (Pacher et al., 2006). Endocannabinoids (anandamide and 2-arachidonoyl glycerol) are synthesized by several enzymes, depending on the intracellular Ca2+ concentration, on postsynaptic neurons and metabolized by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (Piomelli, 2003; Ahn et al., 2008). Among the endocannabinoids, anandamide has important roles for the activation of microglia in neuroinflammation (Raboune et al., 2014; Malek et al., 2015). Moreover, a great deal of evidence from preclinical studies indicates that elevating anandamide concentrations through the inhibition of FAAH can mitigate pain and neuroinflammation (Schlosburg et al., 2009). Several FAAH inhibitors, classified as urea, carbamate, and keto-heterocycle derivatives, have been developed (Seierstad and Breitenbucher, 2008) and progressed to clinical trials to treat inflammatory pain, cannabis dependence, and schizophrenia (Kathuria et al., 2003; Li et al., 2012).

Subsequently, to further understand the function of FAAH...
and to research drug kinetics in vivo, several positron emission tomography (PET) tracers for FAAH were synthesized based on the inhibitors (Wilson et al., 2011; Rotstein et al., 2014; Kumata et al., 2015; Shimoda et al., 2015, 2016) (Fig. 1).

PET is frequently used as an imaging modality or quantification tool for basic and clinical research to elucidate drug kinetics, molecular density, and distribution in vivo. In general, PET studies with reversible-type radiotracers can acquire the nondisplaceable binding potential (BPND) as a reasonable index to estimate receptor density (Innis et al., 2007), which permits pharmacological applications, such as the measurement of dose-occupancy relationships for drugs (Saijo et al., 2009). To the best of our knowledge, although two reversible-type PET tracers for FAAH (Fig. 1A) have been developed, both tracers failed to estimate sufficient BPND values (Liu et al., 2013; Wang et al., 2016). In contrast, PET studies with the irreversible-type radiotracer were generally conducted using two-tissue compartment model analysis with three parameters [k4 = 0, two-tissue compartment model for the irreversible-type radiotracer (2TCMi); Fig. 2]. Of the three parameters, the k3 consisted of Bmax multiplied by the association rate constant (kon) to the target molecule. Thus, estimated k3 gives the most important information for target molecules. Unfortunately, a directly estimated k3 is usually unstable because of including moderate stochastic varieties. Therefore, the macro parameter [e.g., net uptake value (K)] is often estimated as the stable quantitative index for target molecules in place of BPND value (Egerton et al., 2010; Carter et al., 2012; Rusjan et al., 2013; Frick et al., 2015).

Recently, we have developed N-(3,4-dimethylisoxazol-5-yl) piperazine-4-[4-[(2-fluoro-4-[11C]methylphenyl)thiazol-2-yl]-1-carboxamide ([11C]DFMC) (Fig. 1B), which has higher affinity for FAAH (IC50 = 6.1 nM) than the primary PET tracer [11C-carbonyl]-6-hydroxy-(1,1′-biphenyl)-3-yl cyclohexylcarbamate ([11C]CURB) (IC50 = 30 nM). Moreover, [11C]DFMC showed high uptake in rat brain, and the radioactivity was irreversibly trapped (Shimoda et al., 2016).

Materials and Methods

General

All chemical reagents and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO), FUJIFILM Wako Pure Chem. (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan) and used without further purification. The commercially available compound URB597 (>98% purity) was purchased from Sigma-Aldrich and dissolved in saline containing 10% ethanol and 5% Tween 20 for animal experiments. [11C] was produced using a cyclotron (CYPRIS HM-18; Sumitomo Heavy Industries, Tokyo, Japan). All radioactive values were used with decay correction (a half-life of 11C: 20.4 minutes) (Lederer et al., 1967).

Subjects

Male Sprague-Dawley rats (7–10 weeks old, n = 30) were purchased from Japan SLC (Shizuoka, Japan), housed in a temperature-controlled environment with a 12-hour light/dark cycle, and fed a standard diet. All animal experiments were performed according to the recommendations specified by the Committee for the Care and Use of Laboratory Animals of the National Institutes for Radiological Science and Technology and Animal Research: Reporting of In Vivo Experiments guidelines.

Radiotracer

[11C]DFMC was synthesized according to a previous report (Shimoda et al., 2016). Briefly, [11C]DFMC was synthesized using a C-11 coupling reaction of an aryl boronic ester precursor with [11C]methyl iodide in the presence of a Pd catalyst. Over 370 MBq of [11C]DFMC was obtained with radiochemical purity of >99% and molar activity of >37 GBq/μmol.

PET Study

PET Analysis with Blood Sampling. Prior to the PET scan, a rat (n = 4; 509 ± 14 g) was implanted with a polyethylene catheter (FR2; Imamura, Tokyo, Japan) inserted into the left femoral artery for blood sampling. Subsequently, the rat was secured in a custom-designed chamber and placed in a small-animal PET...
scanner (Inveon; Siemens Medical Solutions, Knoxville, TN). Body temperature was maintained using a 40°C water circulation system (TP/Pump TP401; Gaymar Industries, Orchard Park, NY). A 24-gauge intravenous catheter (Terumo Medical Products, Tokyo, Japan) was placed into the tail vein of the rat. A bolus of [11C]DFMC (1 ml, 52–57 MBq, 0.3–0.9 nmol) was injected at a flow rate of 0.5 ml/min via a tail vein catheter. Dynamic emission scans in three-dimensional list mode were performed for 90 minutes (10 second × 12 frames, 20 seconds × 3 frames, 30 seconds × 3 frames, 60 seconds × 3 frames, 150 seconds × 3 frames, and 300 seconds × 15 frames). The acquired PET dynamic images were reconstructed by filtered back projection using a Hanning's filter with a Nyquist cutoff of 0.5 cycle/pixel. The time-activity curves (TACs) of [11C] DFMC were acquired from volumes of interest in the cingulate cortex, striatum (caudate/putamen), hippocampus, thalamus, hypothalamus, pons, and cerebellum by referring to a rat brain magnetic resonance imaging (MRI) template using PMOD software (version 3.4; PMOD technology, Zurich, Switzerland). The radioactivity was decay corrected to the injection time and is expressed as the standardized uptake value (SUV).

For the blocking study, a rat (281 g) culled as described above was intravenously injected with URB597 at a concentration of 3 mg/kg (0.28 ml vehicle) via the tail vein catheter while under anesthesia. It was intravenously injected with URB597 at a concentration of 3 mg/kg (0.28 ml vehicle) via the tail vein catheter while under anesthesia. Dynamic emission scans in three-dimensional list mode were performed for 90 minutes (10 second × 12 frames, 20 seconds × 3 frames, 30 seconds × 3 frames, 60 seconds × 3 frames, 150 seconds × 3 frames, and 300 seconds × 15 frames). The acquired PET dynamic images were reconstructed by filtered back projection using a Hanning's filter with a Nyquist cutoff of 0.5 cycle/pixel. The time-activity curves (TACs) of [11C] DFMC were acquired from volumes of interest in the cingulate cortex, striatum (caudate/putamen), hippocampus, thalamus, hypothalamus, pons, and cerebellum by referring to a rat brain magnetic resonance imaging (MRI) template using PMOD software (version 3.4; PMOD technology, Zurich, Switzerland). The radioactivity was decay corrected to the injection time and is expressed as the standardized uptake value (SUV).

For the blocking study, a rat (281 g) culled as described above was intravenously injected with URB597 at a concentration of 3 mg/kg (0.28 ml vehicle) via the tail vein catheter while under anesthesia. After 30 minutes of anesthesia, a PET assessment with [11C]DFMC (56 MBq; 1.6 nmol) was conducted as described above. Volumes of interest were drawn on the spinal cord in addition to general regions. For counting radioactivity, blood samples were manually collected into microtubes containing heparin (1 μl) at intervals of 20 seconds (0.05 ml for 120 seconds) and 0.5 (0.05 ml for 1 minute), 1 (0.05 ml for 2 minutes), 5 (0.08 ml for 10 minutes), 30 (0.3 ml), 60 (0.4 ml), and 90 minutes (0.5 ml) after initiation of the PET scan. Blood samples were centrifuged at 15,000g at 4°C to separate the plasma. The radioactivity in the whole blood and plasma was measured by a 1480 Wizard autogamma scintillation counter (PerkinElmer, Waltham, MA). The radioactivity was corrected for decay. For metabolite analysis, six plasma samples were separated at 1 (0.02 ml), 5 (0.02 ml), 15 (0.05 ml), 30 (0.1 ml), 60 (0.2 ml), and 90 minutes (0.3 ml) after the injection.

Metabolite analysis was performed as described previously (Yamashiki et al., 2014). Briefly, whole blood samples were treated to separate the plasma, which was deproteinized with an equivalent amount of acetonitrile. An aliquot of the supernatant obtained from the plasma was analyzed using a high-performance liquid chromatography system with a radiation detector (Takai et al., 2001). Plasma protein binding was not determined in our study. The time curves for a fraction of unchanged [11C]DFMC in the plasma were fitted using three exponential equations and subsequently used for kinetic analyses.

Test-Retest PET Studies. Four rats were used twice within 7 days (285 ± 7 g at first and 323 ± 9 g at the second scan) for PET assessments with [11C]DFMC (47–61 MBq; 0.5–0.8 nmol), and the reliability of the data was assessed using the intraclass correlation coefficient (ICC). The parameters were calculated as follows:

1. Relative difference (%) = (scan 2 – scan 1)/scan 1 × 100
2. Test-retest variability (%) = |scan 2 – scan 1|/[(scan 2 + scan 1)/2] × 100
3. Percentage of coefficients of variation (%COV) = S.D./mean × 100
4. ICC with BSMSS as “mean sum of squares between subjects” and WSMSS as “mean sum of squares within subjects”: ICC = (BSMSS – WSMSS)/(BSMSS + WSMSS). An ICC value of –1 indicates no reliability, whereas a value of 1 indicates maximum reliability (Elmenhorst et al., 2012).

Theory

Compartment Model Analysis for Irreversible-Type PET Tracers. To estimate kinetic parameters in PET with [11C]DFMC, 2TCMi (Fig. 2) was conducted. Each rate constant was derived from the following equations:

\[ K_1 = F_E, \]  
\[ k_2 = K_{1/V_d} = FE/V_d, \]  
\[ k_3 = f_{ND}k_{0x}B_{max}, \]

in which \( K_1 \) describes the influx rate of radiotracer from the plasma compartment (CP) to the free and nonspecific compartment (C1); \( k_2 \) represents the efflux rate of radioligand from C1 to CP; \( k_3 \) describes the transfer from C1 to the specific-bound compartment (C2). F is the blood flow, E is the first pass extraction factor, \( V_d \) is the distribution volume of the radiotracer in the C1 compartment, \( f_{ND} \) is the tissue-free fraction, \( k_{0x} \) is the [11C]DFMC-FAAH association rate constant, and \( B_{max} \) is the concentration of FAAH. In addition, the \( K_1 \) as the quantitative index for the net uptake volume of [11C]DFMC with FAAH was determined as follows:

\[ K_{REF} = \frac{k_1k_3}{k_2 + k_3} \]

To compare the accuracy of \( K_1 \) values based on 2TCMi (\( K_{1TCMi} \)), Patlak graphical analysis (PGA) (Patlak et al., 1983) with linear regression was also performed (the slope of a regression line in PGA theoretically equals the \( K_1 \) value).

A Reference Tissue Model for Irreversible-Type PET Tracers. When reference tissue can be employed, the application of PGA as a reference method (PGA reference model (PGAref)) is possible (Patlak and Blasberg, 1985). In this case, the procedure merely replaces \( C_B(t) \) by TAC in the reference tissue. In accordance with a previous report (Patlak and Blasberg, 1985), the slope in graphical analysis reflects the following relation:

\[ \text{Slope} = K_{REF} = \frac{k_1k_3}{k_2 + k_3} \times \frac{k_3}{K_1(1 + K_{ND})} \]

in which, \( K_1' \), \( k_2' \), and \( K_{ND}' \) indicate input rate, output rate, and equilibrium constant in the reference area, respectively. In the reference region without irreversible binding, it may be reasonable to assume that \( K_{ND} = 0 \) (Patlak and Blasberg, 1985). Here, \( \frac{k_3}{K_1} \) is replaced as \( A \),

\[ K_{REF} = A \times K_1 \]

Noninvasive Estimation of the Alternative \( k_3 \) Value Based on the \( K_{REF} \) Value. In this study, we attempted to noninvasively estimate the alternative \( k_3 \) (defined as calculated \( k_3 \)) values, since the \( K_{REF} \) value is an indirect index for FAAH concentration. Calculated \( k_3 \) is induced by modifying eq. 6.

\[ \text{Calculated} \; k_3 = \frac{K_{REF}k_2}{A_{K_1} - K_{REF}} \]

Here, the regional \( K_1 \) and \( k_2 \) values were fixed by averaged values in 2TCMi analyses (\( n = 4 \)). The constant \( A \) was also displaced as follows:

\[ \text{Constant} \; A = \frac{K_{REF}}{K_1}. \]

Conditions for the Use of Calculated \( k_3 \) Values

In this study, we proposed a calculated \( k_3 \) as a new quantitative index for FAAH concentrations. However, there are several conditions for the use of calculated \( k_3 \), which are as follows:

1. Relative difference (%) = (scan 2 – scan 1)/scan 1 × 100
2. Test-retest variability (%) = |scan 2 – scan 1|/[(scan 2 + scan 1)/2] × 100
3. Percentage of coefficients of variation (%COV) = S.D./mean × 100
4. ICC with BSMSS as “mean sum of squares between subjects” and WSMSS as “mean sum of squares within subjects”: ICC = (BSMSS – WSMSS)/(BSMSS + WSMSS). An ICC value of –1 indicates no reliability, whereas a value of 1 indicates maximum reliability (Elmenhorst et al., 2012).

\[ K_1 = FE, \]  
\[ k_2 = K_{1/V_d} = FE/V_d, \]  
\[ k_3 = f_{ND}k_{0x}B_{max}, \]

\[ K_{REF} = \frac{k_1k_3}{k_2 + k_3} \]
1. To estimate averaged $K_1$, $k_2$, and constant $A$, several repeated PET assessments with blood sampling and compartment model analyses are essential in advance of these estimates.

2. It is required that there are no differences in influx ($K_1$) and efflux ($k_2$) rates of radiotracer between the research subject and the baseline subject.

3. The calculated $k_3$ remains an unstable value and therefore includes a specific variation based on individual differences. To surmount this disadvantage, several samples sizes ($n\geq3$, at least) should be considered.

Although there are several limitations, it would be valuable to consider whether calculated $k_3$ can be adapted because calculated $k_3$ in multidose response assays using PET could be more easily obtained than direct $k_3$ with blood sampling.

Multidose URB597 Treatment-Response Assays

A series of dynamic PET scans ($[^{11}C]DFMC: 38–60$ MBq; $0.3–1.0$ nmol) without blood sampling were performed for each rat ($n=3$ for each dose; $241–319$ g) 30 minutes after administration with different doses of URB597 ($0.003, 0.01, 0.03, 0.1, 0.3, 1,$ and $3$ mg/kg in $0.3$-ml vehicle).

The results of the inhibitory experiments were subjected to non-linear regression analysis using Prism 5 (GraphPad Software, La Jolla, CA), and $ED_{50}$ values of URB597 were calculated using each averaged calculated $k_3$ value of several brain regions (cingulate cortex, striatum, hippocampus, thalamus, and cerebellum).

Statistical Methods

Goodness of fit was evaluated using the Akaike information criterion (Akaike, 1974) and the model selection criterion (Handbook, 1995). Values are given as mean ± S.D. The %COV was estimated from the diagonal of the covariance matrix of the fitting. All data analyses were performed using GraphPad Prism v5.0 (GraphPad Software).

Results

Invasive Quantitative PET Analysis Using $[^{11}C]DFMC$. Figure 3 show representative averaged PET/MRI images (A) and TACs (B) in brain regions ($n=4$). PET images were summed between 0 to 90 minutes of acquisition data. The radioactivity was expressed by SUV. Ce, cerebellum; Ci, cingulate cortex; Hi, hippocampus; Hy, hypothalamus; Po, pons; St, striatum; Th, thalamus.

Figure 4A shows the metabolite-corrected plasma input function of $[^{11}C]DFMC$. The unchanged $[^{11}C]DFMC$ in the arterial plasma peaked at $4.19 \pm 0.72$ SUV 2 minutes after the injection and declined to $0.35 \pm 0.03$ SUV 5 minutes after the injection and $0.04 \pm 0.01$ SUV 90 minutes after the injection. The metabolic rate of $[^{11}C]DFMC$ was relatively slow: $65\%$ of the parent compound remained 30 minutes after the injection, and roughly $30\%$ of the parent compound remained 90 minutes after the injection.

Figure 4B shows TACs with a one-tissue compartment model (1TCM) containing $C_P$ and $C_1$ compartments only, and 2TCMi fitting curves in the cingulate cortex. The 1TCM showed a poorly fitting curve for TACs. Conversely, the fitting curve for 2TCMi showed a good shape and indicated good scores (see Supplemental Table 1) regarding the goodness of fit. Thus, the 2TCMi is an adequate kinetic model for this radiotracer. Detailed full kinetic parameters in brain regions are shown in Table 1. Of the rate constants, the directly estimated $k_3$ values were acquired as $0.08–0.18$ minutes$^{-1}$.
TABLE 1
Kinetic rate constants estimated with 2TCMi in PET with [11C]DFMC (n = 4, mean ± S.D.) The %COV was expressed within parentheses.

<table>
<thead>
<tr>
<th>Regions</th>
<th>K1 (ml·cm⁻³·min⁻¹)</th>
<th>k₃ (min⁻¹)</th>
<th>k₅ (min⁻¹)</th>
<th>k₁/k₂ (ml·cm⁻³·min⁻¹)</th>
<th>k₂/k₃ (ml·cm⁻³·min⁻¹)</th>
<th>K₇ (ml·cm⁻³·min⁻¹)</th>
<th>Patlak K₇ (ml·cm⁻³·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cingulate cortex</td>
<td>0.32 ± 0.06</td>
<td>0.09 ± 0.03</td>
<td>0.18 ± 0.04</td>
<td>3.62 ± 0.69</td>
<td>0.62 ± 0.12</td>
<td>0.21 ± 0.04</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.31 ± 0.06</td>
<td>0.10 ± 0.02</td>
<td>0.16 ± 0.03</td>
<td>3.37 ± 0.75</td>
<td>0.53 ± 0.17</td>
<td>0.20 ± 0.04</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.27 ± 0.05</td>
<td>0.08 ± 0.03</td>
<td>0.16 ± 0.04</td>
<td>3.49 ± 0.88</td>
<td>0.56 ± 0.18</td>
<td>0.18 ± 0.04</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.30 ± 0.05</td>
<td>0.10 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>3.11 ± 0.46</td>
<td>0.42 ± 0.12</td>
<td>0.17 ± 0.04</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.27 ± 0.04</td>
<td>0.13 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>2.07 ± 0.20</td>
<td>0.25 ± 0.07</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.26 ± 0.05</td>
<td>0.11 ± 0.03</td>
<td>0.16 ± 0.03</td>
<td>2.58 ± 0.63</td>
<td>0.42 ± 0.11</td>
<td>0.16 ± 0.03</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Pons</td>
<td>0.25 ± 0.04</td>
<td>0.11 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>2.58 ± 0.54</td>
<td>0.22 ± 0.08</td>
<td>0.12 ± 0.03</td>
<td>0.12 ± 0.03</td>
</tr>
</tbody>
</table>

We attempted to noninvasively estimate an alternative k₃ value (defined as calculated k₃ by inserting measured K₇REF values, constant A, and fixed values (averaged K₁ and k₂ values in 2TCMi). Here, because K₁REF values included a small bias (−0.004) compared with K₁TCMi values as described above (Fig. 5C), we modified eq. 7 as follows:

\[ \text{Calculated } k₃ = \frac{(K₇REF - bias)k₂}{AK₁ - (K₇REF - bias)} \]  

with 4.6%–9.5% COV in the regions of interest. The macro-parameter K₇ values for the quantitative uptake value of [11C]DFMC were obtained as 0.12–0.21 ml·cm⁻³·min⁻¹ with 0.0%–0.8% COV in the investigated brain regions.

Next, to validate the accuracy of the estimated K₇ values based on the compartmental analysis (K₇TCMi), we compared these values with those based on graphical analysis (K₇PGA), as shown in Fig. 4C. The slope of the resulting regression line was almost 1 (0.967) and the r-value was 0.995. These results indicate a high correlation between K₇PGA and K₇TCMi values, which suggests that K₇TCMi values were estimated with high reliability.

Validation Studies for the PGA Reference Model. Figure 5, A and B show representative PET-averaged images (A) and TACs (B) in the cingulate cortex and spinal cord of rats pretreated with or without URB597 (3 mg/kg). Radioactivity in the cingulate cortex of the baseline subject was accumulated at a high level without clearance during the PET scan, which was significantly decreased by pretreatment with URB597. Meanwhile, uptake of radioactivity between the baseline and blocking subjects showed no significant differences (P = 0.106) in the spinal cord (Fig. 5B), which suggests that the spinal cord adequately serves as the reference region.

Subsequently, to noninvasively estimate net uptake values (defined as K₇REF), we performed the PGAREF using the TAC of the spinal cord as a reference region. The validity of K₇REF values was evaluated by comparing with K₇TCMi values. Figure 5C shows the relationship between the averaged K₇REF and K₇TCMi values in baseline subjects (n = 4). The slope (k₂/K₁′, defined as constant A, see eq. 8) of the regression line was 0.075 with high correlation (r = 0.981, P < 0.001) and a small intercept (–0.004).

Additionally, to support the accuracy of K₇REF estimations, the reproducibility of the K₇REF values was evaluated by a test-retest study using PET with [11C]DFMC. Table 2 shows the reproducibility of the test-retest PET study for the estimation of K₇REF. In the cingulate cortex, the FAAH-richest region in the brain, the percentage of variability, ICC, and Pearson's r were 8.8, 0.836, and 0.900, respectively. Additionally, the correlation (Pearson's r) between test and retest outcomes in all areas of interest was 0.891, indicating high reproducibility of K₇REF values after PET with [11C]DFMC.

Estimation for Calculated k₃ Values. The K₇REF value is an indirect index of FAAH concentration due to the macro-parameter K₁, k₃, and k₅ rate constants.
The rate constant $k_3$ for $[\text{11C}]\text{CURB}$ in PET with 2TCl analysis was used to noninvasively estimate the macroparameter $K_i$ value, including $k_3$, using the reference tissue method. Prior to kinetic analysis, a blocking PET study using URB597 (3 mg/kg) was conducted to determine the reference region. The heterogeneous uptake of radioactivity in all brain regions of control subjects showed significant displaceability by pretreatment with URB597, whereas radioactive uptake in the spinal cord exhibited nondisplaceable uptake (Fig. 5). In a pathologic report, FAAH-containing neurons were detected in the cerebral cortex, hippocampus, and Purkinje cells of the cerebellar cortex but not in the spinal cord (Tsou et al., 1998). However, to our knowledge, there are no reports regarding quantitative PET analysis using the spinal cord as a reference region. Therefore, we performed a test-retest PET study to validate the use of the spinal cord as a reference region. The ICC of the $K_{REF}$ value in FAAH-rich regions was 0.681–0.836 (Table 2), which supported relatively high reliability for $K_{REF}$ values in the PET study using $[\text{11C}]\text{DFMC}$ with $P_{\text{GRAD}}$. Meanwhile, a high variability (>40%) was detected in the low-FAAH regions ($K_{REF} < 0.005$ minutes$^{-1}$), such as the hypothalamus and pons. The $K_{REF}$ value was concisely estimated with high reproducibility in FAAH-rich regions.

Another concern is the differences in distribution volumes ($K_i/k_2$) between the region of interest and the reference region. Theoretically, the $K_{REF}$ value equals $k_2 K_i / (k_2 + k_3)$ in the case of $K_i / k_2 = K_i / k_2$ (see eq. 5). However, in this study, the distribution of radioactivity in the brain, and the calculated $k_3$ was close to zero. Thus, the ED50 value for URB597 was estimated as a direct index of FAAH concentration.

**Figure 5D** exhibits the relationship between averaged calculated $k_3$ and the directly estimated $k_3$ values. Although both $k_3$ values were robust, the slope of the regression line was 1.177 and showed high correlation ($r = 0.946$, $P = 0.001$). This result suggested that the calculated $k_3$ value would be useful as a direct index of FAAH concentration.

**Estimation for ED50 of URB597 Using Calculated $k_3$**. Figure 6 shows representative PET/MRI images (A) of rat brains treated with multiple doses of URB597 and dose responses of calculated $k_3$ values (B) in the cingulate cortex, striatum, hippocampus, thalamus, and cerebellum. Radioactivity in all brain regions was gradually decreased by increasing the URB597 doses (Fig. 6A). Treatment with 1 mg/kg URB597 almost completely blocked the accumulation of radioactivity in the brain, and the calculated $k_3$ was close to zero. Thus, the ED50 value for URB597 was estimated to be 66.4 $\mu$g/kg in rat brain (Fig. 6B).

**Discussion**

In our study, $[\text{11C}]\text{DFMC}$, the most recently developed PET tracer for FAAH imaging, was used for the index measurement reflecting FAAH concentrations in the brain. The first quantitative PET study for FAAH was performed using $[\text{11C}]\text{CURB}$, a primary PET tracer for FAAH. In that report, the rate constant $k_3$ for $[\text{11C}]\text{CURB}$ in PET with 2TCl analysis was under 0.06 minutes$^{-1}$ in the human brain (Rusjan et al., 2013). 6-Hydroxy-[1,1'-biphenyl]-3-yl cyclohexylcarbamate is a URB597 derivative and has lower affinity ($IC_{50} = 7.7$ nM) (Clapper et al., 2009). $N$-(3,4-Dimethylisoxazol-5-yl)-4-(2-fluoro-4-methylphenyl) thiazol-2-yl)piperazine-1-carboxamide has been recently developed as an inhibitor possessing a 3-fold higher affinity for FAAH than URB597 ($IC_{50} = 30$ nM) (Giang and Cravatt, 1997; Maccarrone et al., 1998). Thereafter, the ED50 value for URB597 was estimated as a direct index of FAAH concentration.

**Table 2**

<table>
<thead>
<tr>
<th>Region</th>
<th>$K_{REF}$ value (min$^{-1}$)</th>
<th>Relative Difference (%) (Mean ± S.D.)</th>
<th>%Variability (Mean ± S.D.)</th>
<th>ICC</th>
<th>Pearson’s r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cingulate cortex</td>
<td>0.0108 ± 0.0021</td>
<td>-7.3 ± 10.3</td>
<td>8.8 ± 10.8</td>
<td>0.936</td>
<td>0.900</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.0092 ± 0.0027</td>
<td>5.3 ± 21.4</td>
<td>11.8 ± 13.6</td>
<td>0.835</td>
<td>0.909</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.0095 ± 0.0025</td>
<td>1.3 ± 22.5</td>
<td>15.0 ± 10.9</td>
<td>0.681</td>
<td>0.892</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.0081 ± 0.0023</td>
<td>-1.5 ± 20.0</td>
<td>14.8 ± 9.1</td>
<td>0.700</td>
<td>0.976</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.0045 ± 0.0018</td>
<td>7.5 ± 76.8</td>
<td>47.2 ± 36.3</td>
<td>0.430</td>
<td>0.226</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.0080 ± 0.0024</td>
<td>2.9 ± 24.9</td>
<td>15.3 ± 14.4</td>
<td>0.735</td>
<td>0.759</td>
</tr>
<tr>
<td>Pons</td>
<td>0.0040 ± 0.0015</td>
<td>-18.8 ± 41.7</td>
<td>45.5 ± 23.7</td>
<td>0.369</td>
<td>0.304</td>
</tr>
</tbody>
</table>

Fig. 6. Dose-response assay using URB597. (A) Representative PET/MRI images of $[\text{11C}]\text{DFMC}$ in the brains of rats treated with different doses (0.01, 0.1, 0.3, and 1 mg/kg) of URB597. (B) The relationship between calculated $k_3$ values (minute$^{-1}$) and doses of URB597 in various brain regions. Averaged values of calculated $k_3$ in the cingulate cortex, striatum, hippocampus, thalamus, and cerebellum are plotted against the dose of URB597. Ce, cerebellum; Ci, cingulate cortex; Hi, hippocampus; St, striatum; Th, thalamus.
volume in the spinal cord (Kf/k2) did not equal K3/k2 in other brain regions (see in Supplemental Table 2). Therefore, the KREF values are modified to Kf,k3(k2+k3)/K3. In the results of the PAGAREF analysis, the KREF values in brain regions were estimated with a range of 0.004–0.011 minutes⁻¹. Compared with the K2CTCMI values, the KREF values showed high correlation (r > 0.95; Fig. 5C). In this regression, the slope showed 0.075 (= k3/K3), which is defined as constant A in eq. 8. However, a small negative bias (−0.004) of the KREF value was recognized, although PAGAREF and K2CTCMI are proportional in theory. This small negative bias may be caused by the slight accumulation of the radiotracer at nondisplaceable sites in the spinal cord, which would cause an undesirable increase in the value of TAC in the spinal cord. Nevertheless, the spinal cord would be an adequate reference region for the estimation of regional KREF values in the present PAGAREF analysis. Moreover, estimated KREF values would be a reasonable index for alternative net uptake values of [11C]DFMC with FAAH despite including a small bias.

Finally, to propose a pharmacological application of [11C]DFMC-PET using KREF values, we estimated ED50 values of URB597 in the brain in vivo. URB597 has been developed a decade before (Fegley et al., 2005), and widely tested as a treatment of neuroinflammation and pain (Murphy et al., 2012; Lamazzo et al., 2015). Here, since the KREF value is not proportional to the regional FAAH concentrations in theory (eqs. 3 and 6), we tentatively estimated the k3 value (defined as calculated k3) from KREF value (eq. 9). In a comparison of the directly estimated k3 obtained by the compartmental analysis with blood sampling, the calculated k3 showed high correlation (r = 0.946), although a slight overestimation was exhibited (Fig. 5D). This result suggested that the calculated k3 would be favorably used as an alternative parameter of the directly estimated k3 value, which motivated us to progress our pharmacological application study. However, there is a considerable limitation in the use of calculated k3. Since the equation for calculated k3 includes two variable parameters, Kf and k2, it is important that administration of URB597 does not affect blood flow. Fortunately, administration of under 1 mg/kg (the maximum dose used in this assessment) of URB597 did not produce any effects on the initial uptake of radioactivity (Supplemental Fig. 1). Therefore, the present dose-response assay using the calculated k3 would be expected to give reasonable ED50 values of URB597 in brain regions without effects on blood flow.

Although the assay used a calculated-k3, the ED50 values of URB597 were estimated to be 66.4 µg/kg (0.2 µmol/kg ≈ 60 nmol/head) in the rat brain (Fig. 6B). Previously, we reported that the in vitro IC50 value of URB597 using 60 nmol/head) in the rat brain (Fig. 6B). Previously, we estimated ED50 values of URB597 using KREF values, we estimated ED50 values of URB597 responsible for FAAH inhibition in rat brain as one of the pharmacological applications in vivo. Thus, our technique using PET with [11C]DFMC could contribute to occupancy studies using rats for new pharmaceuticals for the treatment of central nervous system disorders targeting FAAH.

Acknowledgments

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Authorship Contributions

Participated in research design: Yamasaki T, Ohya T. Conducted experiments: Yamasaki T, Zhang Y, Wakizaka H. Contributed new reagents or analytic tools: Mori W, Nengaki N, Fujinaga M. Performed data analysis: Yamasaki T. Wrote or contributed to the writing of the manuscript: Yamasaki T, Ohya T, Kikuchi T, Zhang MR.

References

Supplemental Data

Development of an In Vivo Method to Estimate Effective Drug Doses and Quantify Fatty Acid Amide Hydrolase in Rodent Brain using Positron Emission Tomography Tracer $N$-(3,4-dimethylisoxazol-5-yl)piperazine-4-[4-(2-fluoro-4-[11C]methylphenyl)thiazol-2-yl]-1-carboxamide ($[^{11}\text{C}]$DFMC)

Tomoteru Yamasaki, PhD; Tomoyuki Ohya, PhD; Wakana Mori, BS; Yiding Zhang, BS; Hidekatsu Wakizaka, BS; Nobuki Nengaki, BS; Masayuki Fujinaga, PhD; Tatsuya Kikuchi, PhD; Ming-Rong Zhang, PhD

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- Table S2: Comparison of $k_1$ and $k_2$ parameters between the baseline and blocking subjects.
- Figure S1: Time-activity curves of [$^{11}$C]DFMC in the whole brain of rats treated with 3, 1, 0.3, 0.03, or 0.003 mg/kg of URB597.
Table S1. Results of goodness-of-fit assessments (AIC, SC, MSC, and R square) in the cingulate cortex.

<table>
<thead>
<tr>
<th>Kinetic model</th>
<th>AIC</th>
<th>SC</th>
<th>MSC</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td>1TCM</td>
<td>27.7 ± 6.1</td>
<td>31.0 ± 6.1</td>
<td>3.67 ± 0.20</td>
<td>0.977 ± 0.004</td>
</tr>
<tr>
<td>2TCMi</td>
<td>-47.4 ± 20.1</td>
<td>-42.4 ± 20.1</td>
<td>5.61 ± 0.52</td>
<td>0.996 ± 0.001</td>
</tr>
</tbody>
</table>
Table S2. Comparison of K₁ and k₂ parameters between the baseline and blocking (3 mg/kg URB597) subjects.

<table>
<thead>
<tr>
<th>Regions</th>
<th>K₁ (mL·cm⁻³·min⁻¹)</th>
<th>k₂ (min⁻¹)</th>
<th>K₁/k₂ (mL·cm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (n = 4)</td>
<td>Baseline (n = 4)</td>
<td>Baseline (n = 4)</td>
</tr>
<tr>
<td></td>
<td>Blocking (n = 1)</td>
<td>Blocking (n = 1)</td>
<td>Blocking (n = 1)</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>0.32 ± 0.06</td>
<td>0.34</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(1.7 ± 0.1)</td>
<td>1.4</td>
<td>(12.9 ± 2.3)</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.31 ± 0.06</td>
<td>0.35</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.2)</td>
<td>1.4</td>
<td>(9.7 ± 1.5)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.27 ± 0.05</td>
<td>0.34</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(1.1 ± 0.1)</td>
<td>1.4</td>
<td>(9.0 ± 1.5)</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.30 ± 0.05</td>
<td>0.38</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(1.2 ± 0.2)</td>
<td>1.3</td>
<td>(7.3 ± 1.7)</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.27 ± 0.04</td>
<td>0.35</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(1.8 ± 0.3)</td>
<td>2.4</td>
<td>(8.5 ± 1.9)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.26 ± 0.05</td>
<td>0.41</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(1.4 ± 0.1)</td>
<td>1.7</td>
<td>(8.9 ± 1.3)</td>
</tr>
<tr>
<td>Pons</td>
<td>0.28 ± 0.04</td>
<td>0.39</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(1.2 ± 0.2)</td>
<td>2.3</td>
<td>(5.6 ± 1.2)</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.14 ± 0.02</td>
<td>0.26</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>(2.5 ± 0.1)</td>
<td>(5.4)</td>
<td>(9.6 ± 0.5)</td>
</tr>
</tbody>
</table>
Figure S1. Averaged (n = 3) time-activity curves of $[^{11}C]$DFMC in the whole brain of rats treated with 3, 1, 0.3, 0.03, or 0.003 mg/kg of URB597.