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Synthesis and evaluation of ^{18}F-labeled mitiglinide derivatives as positron emission tomography tracers for β-cell imaging

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ABSTRACT

Measuring changes in β-cell mass in vivo during progression of diabetes mellitus is important for understanding the pathogenesis, facilitating early diagnosis, and developing novel therapeutics for this disease. However, a non-invasive method has not been developed. A novel series of mitiglinide derivatives (*o*-FMIT, *m*-FMIT and *p*-FMIT; FMITs) were synthesized and their binding affinity for the sulfonylurea receptor 1 (SUR1) of pancreatic islets were evaluated by inhibition studies. (+)-(*S*)-*o*-FMIT had the highest affinity of our synthesized FMITs (IC_{50} = 1.8 μM). (+)-(*S*)-*o*-[^{18}F]FMIT was obtained with radiochemical yield of 18% by radiofluorination of racemic precursor **7**, hydrolysis, and optical resolution with chiral HPLC; its radiochemical purity was >99%. In biodistribution experiments using normal mice, (+)-(*S*)-*o*-[^{18}F]FMIT showed 1.94 ± 0.42% ID/g of pancreatic uptake at 5 min p.i., and decreases in radioactivity in the liver (located close to the pancreas) was relatively rapid. Ex vivo autoradiography experiments using pancreatic sections confirmed accumulation of (+)-(*S*)-*o*-[^{18}F]FMIT in pancreatic β-cells. These results suggest that (+)-(*S*)-*o*-[^{18}F]FMIT meets the basic requirements for an radiotracer, and could be a candidate positron emission tomography tracer for in vivo imaging of pancreatic β-cells.

1. Introduction

The International Diabetes Federation has reported that the total number of people with diabetes mellitus will rise from 285 million in 2009 to 435 million in 2030. There are two major types of diabetes mellitus: type-1 diabetes (T1D) and type-2 diabetes (T2D).[1] T1D is characterized by selective destruction of β-cells by the autoimmune reaction, and pancreatic β-cell mass (BCM) in T1D is already lost at disease onset. T2D is characterized by the decreased ability to secrete insulin and increased insulin resistance, it also has been reported that BCM in T2D is decreased significantly.[2–5] Recently, incretin-related drugs were reported to have proliferative and anti-apoptotic effects on pancreatic β-cells in in vitro or rodent experiments.[6–8] However, to date, non-invasive quantification of BCM has not been possible, so how and when a decrease in BCM begins and whether incretin-related drugs preserve BCM in humans is not known. Therefore, a non-invasive method for BCM measurement is required urgently for understanding the pathogenesis, facilitating early diagnosis, and developing novel therapeutics for diabetes.

Sulfonylurea (SU) agents are insulin secretagogues, and are used widely in T2D treatment. They close the adenosine triphosphate-sensitive potassium ion channel (K_{ATP} channel) in the pancreatic β-cell membrane, thereby depolarizing the cell and triggering insulin secretion.[9] The K_{ATP} channel is an octameric complex of two protein subunits, an inwardly rectifying K^+ (Kir6.x) ion channel and a sulfonylurea receptor (SUR), which is the target for SU agents.[9,10] The pancreatic β-cell K_{ATP} channel is composed of Kir6.2 and SUR1 subunits.[11] Other K_{ATP} channels comprise Kir6.2 and SUR2A (heart muscle and skeletal muscle), Kir6.2 and SUR2B (smooth muscle), and Kir6.1 and SUR2B (vascular smooth muscle).[9] In recent years, SUR2B expression in human liver tissue has been described.[12]

Several radiolabeled tracers based on the core structure of SU agents have been developed as imaging probes for SUR1 (Fig. 1)

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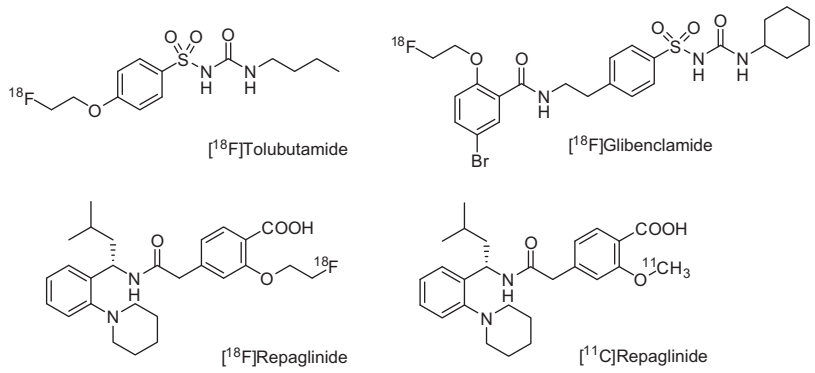


Figure 1. Chemical structures of radioligands for imaging of SUR1.

in recent years.[13–18] However, these imaging probes are not available because of low uptake in pancreatic tissue and high uptake in liver tissue located close to the pancreas. Promising candidates for pancreatic β-cell imaging tracers based on SU agents need to have relatively low lipophilicity and have high affinity for SUR1. In this context, mitiglinide is a promising candidate because of its low lipophilicity (calculated log*D* (*c*log*D*) value (pH 7.4) for mitiglinide, −0.29; glibenclamide, 1.14; repaglinide, 1.99) and high affinity and selectivity for SUR1 (SUR1/Kir6.2:3.8 nM, SUR2A/Kir6.2:3200 nM, SUR2B/Kir6.2:4600 nM).[19]

We synthesized a novel series of mitiglinide derivatives, 2-(fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydroisoindolin-2-yl)-4-oxobutanoic acid (FMITs) and 2*S*-(2-[^{18}F]fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydroiso-indolin-2-yl)-4-oxobutanoic acid [(+)-(*S*)-*o*-[^{18}F]FMIT] and evaluated their potential as in vivo pancreatic β-cell imaging tracers (Fig. 2).

2. Results and discussion

2.1. Chemistry

The synthesis of mitiglinide derivatives containing fluorine (*o*-, *m*- and *p*-FMIT) is shown in Scheme 1. The succinic acid derivatives **2a–c** were prepared, using a method based on the synthetic strategy reported by Kamijo and co-workers[20] by cross-aldol condensation of the benzaldehydes **1a**, **b**, and **c** (which have a benzyloxy group at the *o*-, *m*-, and *p*-position, respectively) with dimethyl succinate in the presence of potassium hydroxide, followed by hydrolysis of the ester. Acids **2a–c** were treated with acetic anhydride in dichloromethane to afford the cyclic acid anhydride derivatives, followed by amidation with 3*a*,4,7,7*a*-tetrahydroisoindoline (**3**) and successive esterification to afford the amides **4a–c**. Catalytic hydrogenation of **4a–c** gave the phenols **5a–c**.

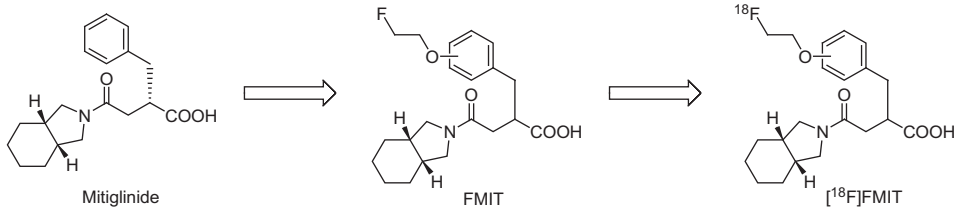
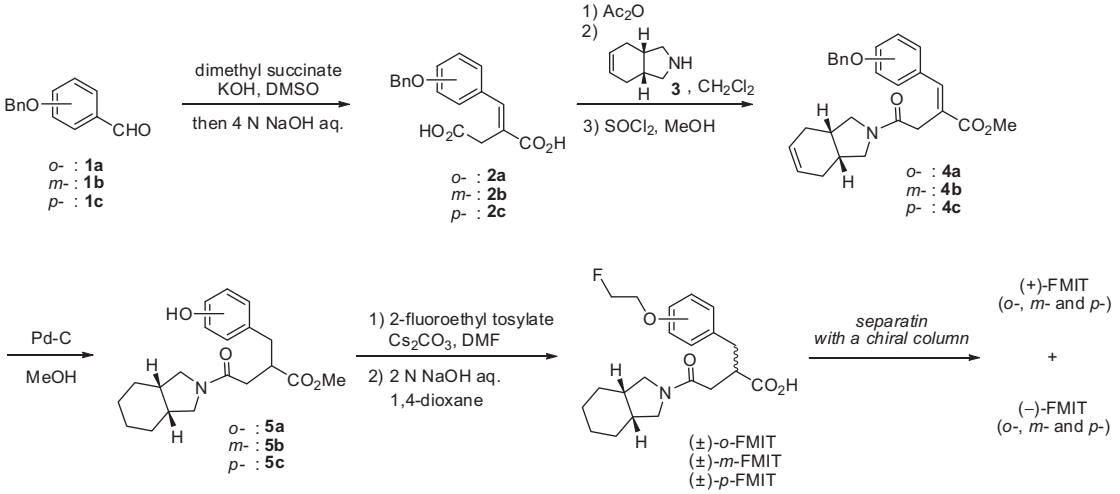


Figure 2. Structure of mitiglinide and ^{18}F-labeled mitiglinide derivatives.



Scheme 1. Syntheses of mitiglinide derivatives (+)- and (−)-FMIT.

Finally, **5a–c** were alkylated with 2-fluoroethyl tosylate in the presence of cesium carbonate in DMF, followed by saponification with aqueous sodium hydroxide in 1,4-dioxane to give (±)-FMIT. Optical resolution using HPLC with a chiral column gave enantiomerically pure (+)-FMIT and (−)-FMIT (*o*-, *m*- and *p*-, respectively).

2.2. In vitro binding assays

The FMITs were evaluated using dispersed islet cells from ddY mice pancreas. The binding of [^{3}H]glibenclamide was displaced by (+)-*o*-FMIT, (+)-*m*-FMIT, and (−)-*p*-FMIT in a concentration-dependent manner, similar to mitiglinide Ca^{2+} (Fig. 3). The results of these assays are summarized in Table 1. IC_{50} values of (+)-*o*-FMIT, (+)-*m*-FMIT, and (−)-*p*-FMIT were 1.8, 14.0, and 10.7 μM, respectively. Namely, (+)-*o*-FMIT had the highest binding affinity for pancreatic islet SUR1 in our synthesized FMITs, but its affinity was lower than that of mitiglinide. The other enantiomers, (−)-*o*-FMIT, (−)-*m*-FMIT and (+)-*p*-FMIT, did not show inhibitory potency.

2.3. Stereochemistry

The absolute configuration of (+)-*o*-FMIT and (−)-*o*-FMIT was determined by ^{1}H NMR spectra using corresponding α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) esters with Eu(fod)$_3$.[21–24] (+)-*o*-FMIT and (−)-*o*-FMIT were transformed into corresponding diastereomeric esters **6** by reduction of the carboxyl group and esterification according to the method of Dale et al.[25,26] The magnitude of the lanthanide-induced shift (LIS) by Eu(fod)$_3$ for the methoxyl group of **6** in ^{1}H NMR indicated that the configuration of (+)-*o*-FMIT was *S* and that of (−)-*o*-FMIT was *R* (Scheme 2). The result of the binding affinity for pancreatic β-cell SUR1 of each enantiomer suggested that the *S*-configuration of *o*-FMIT (which has the same absolute configuration as mitiglinide) was important for binding affinity.

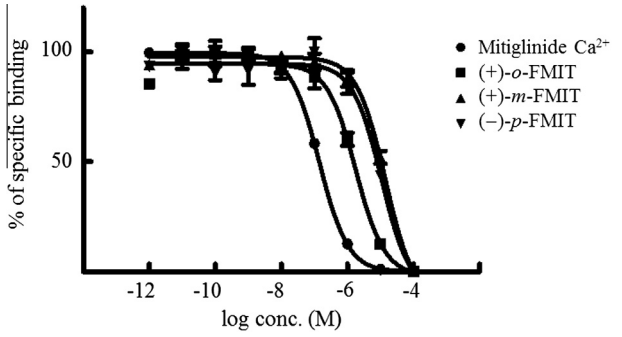


Figure 3. Inhibition of [^{3}H]glibenclamide binding to islet cells at various concentrations of (+)-*o*-FMIT, (+)-*m*-FMIT, and (−)-*p*-FMIT. Specific binding of [^{3}H]glibenclamide and displacement curves by FMITs are shown. Data are the mean ± SD (n = 4).

Table 1
Binding inhibition study of mitiglinide and FMITs with [^{3}H]glibenclamide

Compound		t_R[a] (min)	IC_{50}[b] (μM)
Mitiglinide		—	0.14 (0.12–0.17)
o-FMIT	(+)-	11.6	1.8 (1.2–2.6)
	(−)-	24.3	N.D.
m-FMIT	(−)-	12.5	14.0 (10.8–18.1)
	(+)-	44.6	N.D.
p-FMIT	(−)-	17.4	10.7 (7.0–16.1)
	(+)-	28.8	N.D.

[a] Retention time using chiral HPLC (CHIRALPAK AY-H, 10 × 250 mm, stationary phase acetonitrile/methanol/TFA = 95/5/0.1, flow rate 2.0 mL/min).

[b] Best-fit values and 95% confidence intervals of 50% inhibitory concentrations obtained from displacement of the [^{3}H]glibenclamide assay. R^2 values were >0.85 in experiments used to determine the binding specificity of each compound.

2.4. Radiochemistry

The preparation of precursor **7** and its radiofluorination, shown in Scheme 3, were undertaken to obtain ^{18}F-labeled (+)-(*S*)-*o*-FMIT. The phenol **5a**, with a phenolic hydroxyl group at the *o*-position, was alkylated with 2-bromoethyl tosylate to give the bromo derivative **7**. Although we attempted to label the chiral **7** (given by optical resolution) with ^{18}F, we found that the resulting *o*-[^{18}F]FMIT was racemized. Therefore, racemic **7** was used as a precursor, treated with [^{18}F]KF and Kryptofix$_{222}$ in acetonitrile at 140 °C in a sealed tube, to afford the ^{18}F-labeled methyl ester. This was saponified successively with an aqueous solution of sodium hydroxide in 1,4-dioxane. Then, optical resolution using HPLC with a chiral column of the racemic *o*-[^{18}F]FMIT gave the enantiomerically pure *o*-[^{18}F]FMIT (Fig. 4). The radiochemical identity of *o*-[^{18}F]FMIT was verified by co-injection HPLC analyses with nonradioactive *o*-FMIT. Each chiral (+)-(*S*)-*o*-[^{18}F]FMIT showed a single radioactive peak at the same retention time as corresponding nonradioactive *o*-FMIT. The radiochemical yields of (+)-(*S*)-*o*-[^{18}F]FMIT and (−)-(*R*)-o-[^{18}F]FMIT were 18% and 13%, respectively. Both of their radiochemical purities after purification by HPLC with a chiral column were >99%.

2.5. Biodistribution studies

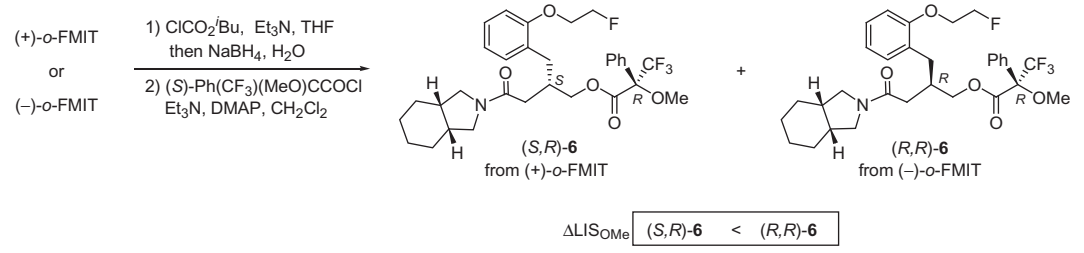
The tissue time-course distributions of (+)-(*S*)-*o*-[^{18}F]FMIT in male ddY mice are summarized in Table 2. (+)-(*S*)-*o*-[^{18}F]FMIT showed 1.94 ± 0.42% ID/g of pancreatic uptake at 5 min p.i. and radioactivity washout from the pancreas was relatively slow (1.45 ± 0.19% ID/g at 60 min p.i.). Radioactivity in the liver showed rapid clearance (20.83 ± 1.35% ID/g at 5 min p.i. and 2.18 ± 0.34% ID/g at 120 min p.i.). Radioactivity in the kidney showed rapid clearance (7.77 ± 3.30% ID/g at 5 min p.i. and 1.33 ± 0.30% ID/g at 120 min p.i.). In addition, radioactivity in the bone was low, suggesting that (+)-(*S*)-*o*-[^{18}F^{18}F]FMIT was relatively stable to in vivo defluorination.

In the case of (−)-(*R*)-*o*-[^{18}F]FMIT, maximum uptake in the pancreas was observed at 5 min p.i. and was 1.18 ± 0.30% ID/g. Comparison of the two *o*-[^{18}F]FMITs at 5, 15 and 60 min p.i. revealed the amount of uptake of (+)-(*S*)-*o*-[^{18}F]FMIT in the pancreas to be approximately double that of (−)-(*R*)-*o*-[^{18}F]FMIT (Fig. 5). The difference derived from the presence or absence of the binding affinity for SUR1 would suggest the specific binding of (+)-(*S*)-*o*-[^{18}F]FMIT. Pancreas-to-liver (P/L), pancreas-to-kidney (P/K), and pancreas-to-blood (P/B) ratios at different time points are shown in Figure 6. The P/L ratio was ⩽0.57 at 60 min, the P/K ratio was ⩽0.81 at 120 min, and the P/B ratio was ⩽0.64 at 60 min.

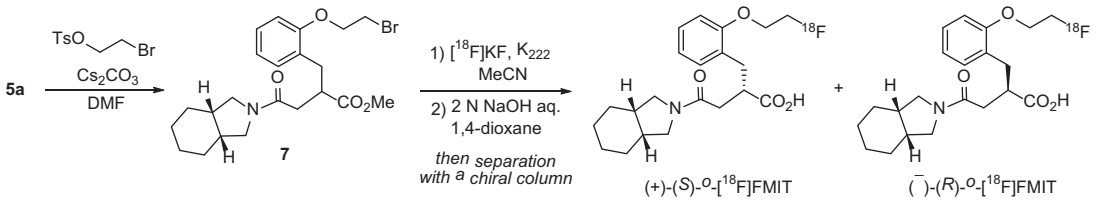
It is considered that the hepatic clearance of (+)-(*S*)-*o*-[^{18}F]FMIT is relatively rapid and that the P/L is high when compared with other radiolabeled compounds based on SU agents.[14–16]

2.6. Ex vivo autoradiography

To further characterize the potential of (+)-(*S*)-*o*-[^{18}F]FMIT as an agent for imaging pancreatic β-cells, we undertook an ex vivo autoradiography study in normal ddY mice (6-week-old, male). Radioactive signals were observed in pancreatic sections, whose localizations were relatively consistent with islets colored by insulin immunostaining on serial sections, which suggested that (+)-(*S*)-*o*-[^{18}F]FMIT accumulated in pancreatic β-cells. However, radioactive signals in other regions were also observed, which suggested non-specific binding of (+)-(*S*)-*o*-[^{18}F]FMIT (Fig. 7).



Scheme 2. Syntheses of MTPA ester (*S*,*R*)-**6** and (*R*,*R*)-**6**.



Scheme 3. Radiosynthesis of (+)-(*S*)- and (−)-(*R*)-*o*-[^{18}F]FMIT.

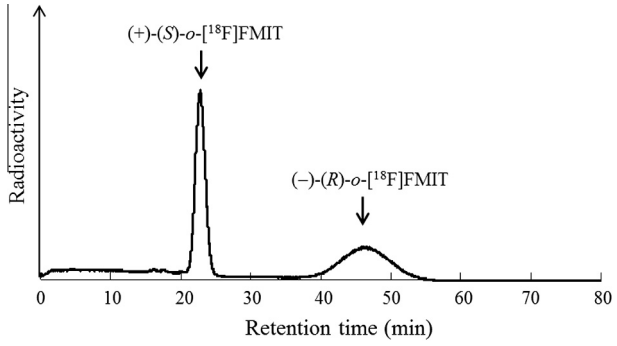


Figure 4. HPLC chromatograms of (+)-(*S*)-*o*-[^{18}F]FMIT and (−)-(*R*)-*o*-[^{18}F]FMIT. Peaks at a retention time of 22.9 min and 46.4 min were (+)-(*S*)-*o*-[^{18}F]FMIT and (−)-(*R*)-*o*-[^{18}F]FMIT, respectively.

3. Conclusions

We synthesized a novel series of mitiglinide derivatives (*o*-FMIT, *m*-FMIT, and *p*-FMIT) and evaluated their binding affinity for pancreatic islet SUR1. (+)-*o*-FMIT showed the highest affinity of our synthesized FMITs, which suggested that introduction of a fluoroethoxy group at the *o*-position affects the affinity less than at other positions. In addition, we determined the absolute configuration of each enantiomer of *o*-FMIT by ^{1}H NMR spectra using MTPA esters with Eu(fod)$_3$, which showed that the exact configuration at the 2-position of FMITs must be especially important to the affinity for SUR1.

We synthesized (+)-(*S*)-*o*-[^{18}F]FMIT and evaluated its potential as an in vivo pancreatic β-cell imaging tracer. In in vivo experiments, (+)-(*S*)-*o*-[^{18}F]FMIT showed pancreatic uptake and relatively rapid clearance in the liver and kidney. The detected localization of the radioactive signals by ex vivo autoradiography corresponded with that of pancreatic β-cells shown by insulin immunostaining.

Further enhancements of pancreatic uptake and specific binding to pancreatic β-cells are required for development of a potential tracer for in vivo imaging of pancreatic β-cells. Nevertheless, these results provide useful information for targeting SUR1 of pancreatic β-cells.

4. Experimental sections

4.1. General

[^{18}F]Fluoride was produced in a cyclotron (CYPRIS HM-18, Sumitomo Heavy Industries Ltd., Tokyo, Japan). Radioactivity was measured with an IGC-7 curiemeter (Aloka, Tokyo, Japan), an Atomlab100+ (Biodex Medical Systems Inc., Shirley, NY, USA) or CRC-15BETA (Capintec Inc., Ramsey, NJ, USA) dose calibrator or a Wallac 1480 WIZARD 3″ (PerkinElmer Inc., Waltham, MA, USA) γ-counter.

Table 2
Tissue distribution of (+)-(*S*)-*o*-[^{18}F]FMIT in ddY mice

Organ or tissue	5 min	15 min	30 min	60 min	120 min
Pancreas	1.94 ± 0.42	1.65 ± 0.15	1.30 ± 0.12	1.45 ± 0.19	1.08 ± 0.17
Blood	3.93 ± 0.76	2.76 ± 0.26	2.07 ± 0.18	2.27 ± 0.28	2.16 ± 0.51
Heart	2.63 ± 0.42	2.16 ± 0.19	1.82 ± 0.21	2.10 ± 0.26	1.72 ± 0.11
Lung	2.81 ± 0.65	2.25 ± 0.20	1.71 ± 0.22	1.84 ± 0.33	1.37 ± 0.14
Stomach	2.22 ± 1.06	5.36 ± 3.32	2.54 ± 0.71	2.07 ± 1.51	2.66 ± 1.47
Intestine	12.25 ± 4.46	26.72 ± 3.65	35.27 ± 4.73	31.79 ± 6.13	13.84 ± 5.08
Liver	20.83 ± 1.35	5.40 ± 0.46	3.44 ± 1.73	2.55 ± 0.20	2.18 ± 0.34
Spleen	1.91 ± 0.28	1.71 ± 0.17	1.36 ± 0.17	1.61 ± 0.30	1.22 ± 0.12
Kidney	7.77 ± 3.30	5.98 ± 1.20	3.16 ± 1.07	2.16 ± 0.48	1.33 ± 0.30
Bone	1.40 ± 0.31	1.22 ± 0.11	1.22 ± 0.08	1.80 ± 0.19	3.01 ± 0.62

Values are % ID/g (mean ± SD) for 5 animals at each interval.

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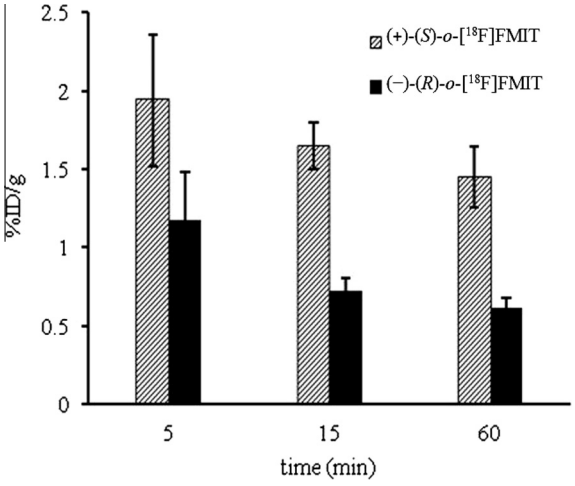

Figure 5. Comparative uptake of (+)-(*S*)- and (−)-(*R*)-*o*-[^{18}F]FMIT at 5, 15 and 60 min p.i. in the pancreas.

^{1}H NMR spectra were obtained on a JEOL JNM-ECS400 (400 MHz) spectrometer (JEOL, Tokyo, Japan). Chemical shifts were reported in ppm using tetramethylsilane or residual solvent as the internal reference. Infrared (IR) spectra were recorded on an FTIR-8300 diffraction grating infrared spectrophotometer (Shimadzu, Kyoto, Japan). Mass spectra (MS) were determined on a JEOL JMS-SX 102A QQ or a JEOL JMS-GC-mate mass spectrometer (JEOL). Silica gel column chromatography was undertaken using a W-Prep 2XY system (Yamazen, Osaka, Japan) using a Hi Flash™ 40 mm 60 Å silica gel column (Yamazen). Kieselgel 60 F-254 plates (0.25 mm; Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). Preparative thin-layer chromatography (PTLC) was conducted with a silica gel 60 F-254 plate (0.5 mm; Merck). An LC-20AD system (Shimadzu) was used for high-performance liquid chromatography (HPLC), with SPD-20A (Shimadzu) ultraviolet (UV; $\lambda = 254$ nm) and NDW-351D (Aloka, Tokyo, Japan) radioisotope (RI) detectors, and a Cosmosil 5C_{18}-AR-II (10 × 250 mm; Nacalai Tesque, Kyoto, Japan) reverse-phase HPLC column and a CHIRALPAK AY-H (10 × 250 mm; Daicel, Tokyo, Japan) chiral HPLC column. Specific rotations were recorded using a SEPA-500 automatic digital polarimeter (Horiba, Kyoto, Japan). All chemicals used were of reagent grade. Calculated log*D* values were obtained by computational methods using ACD/Labs v12.0 (Advanced Chemistry Development, Toronto, ON, Canada).

4.2. Animal experiments

Six-week-old male ddY mice were obtained from Japan SLC (Kyoto, Japan). Animal studies were conducted in accordance with institutional guidelines. Experimental procedures were approved by the Animal Care Committee of Kyoto University (Kyoto, Japan).

4.3. Synthesis

4.3.1. Procedure for the preparation of dicarboxylic acids 2a–c

Potassium hydroxide (2.4 equiv) was added to a solution of *o*-, *m*-, or *p*-benzyloxybenzaldehyde (**1a–c**) and dimethyl succinate (3.0 equiv) in dimethyl sulfoxide, and the mixture stirred for 15 h at 80 °C. An aqueous solution of sodium hydroxide (5 M, 30 mL) was added and the mixture stirred for 5 h at 120 °C. After the reaction, distilled water was added to the reaction mixture, which was then washed with ethyl acetate. The aqueous layer was acidified with 6 N hydrochloric acid, and extracted with ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, and evaporated under vacuum to give a pale-yellow solid. The solid was washed with hot water and dried to afford dicarboxylic acids **2a–c**.

4.3.1.1. 2-(2-Benzyloxybenzylidene)succinic acid (2a). The reaction was carried out with *o*-benzyloxybenzaldehyde (**1a**) (5.00 g, 23.6 mmol) in dimethyl sulfoxide (24 mL) to afford **2a** (2.98 g, 40%). mp 216–217 °C (recrystallized from ethyl acetate). ^{1}H NMR (DMSO-d_6) δ 3.30 (2H, s), 5.17 (2H, s), 7.00 (1H, t, $J = 7.7$ Hz), 7.14 (1H, d, $J = 8.5$ Hz), 7.26–7.45 (7H, m), 7.86 (1H, s), 12.47 (2H, br s). IR (KBr) ν 3042, 2934, 2872, 2540, 2361, 2341, 1697, 1630, 1452, 1420, 1290, 1232 cm^{-1}. EI-MS *m*/*z*: 312 (M^+). HRMS *m*/*z*: 312.1002 (Calcd for $C_{18}H_{16}O_5$: 312.0997). Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.28; H, 5.20.

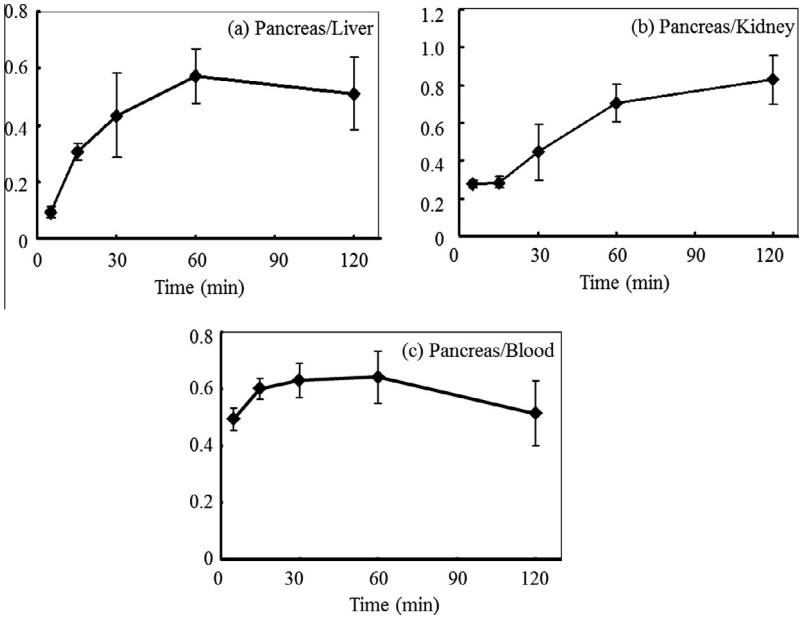

Figure 6. Uptake ratios of (+)-(*S*)-*o*-[^{18}F]FMIT. (a) pancreas/liver, (b) pancreas/kidney, and (c) pancreas/blood. Ratios were calculated based on the % ID/g of each organ.

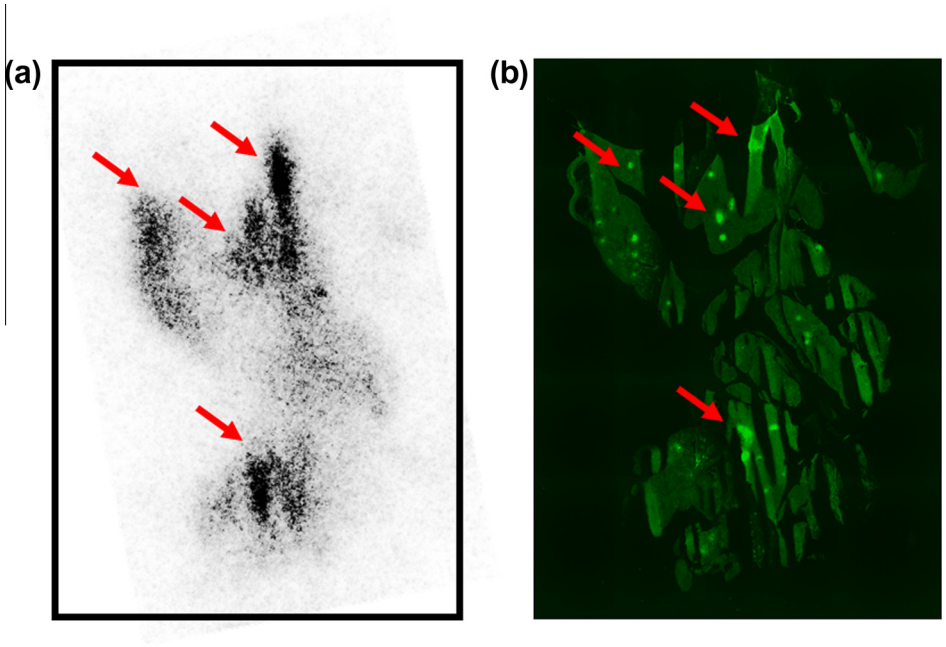

Figure 7. (a) Autoradiography of (+)-(*S*)-*o*-[^{18}F]FMIT. (b) Localization with insulin by immunohistochemistry.

4.3.1.2. 2-(3-Benzyloxybenzylidene)succinic acid (2b). The reaction was carried out with *m*-benzyloxybenzaldehyde (**1b**) (7.50 g, 35.3 mmol) in dimethyl sulfoxide (30 mL) to afford **2b** (3.84 g, 35%). mp 197–198 °C (recrystallized from ethyl acetate). ^{1}H NMR (DMSO-d_6) δ 3.40 (2H, s), 5.16 (2H, s), 7.01–7.09 (3H, m), 7.35–7.50 (6H, m), 7.75 (1H, s), 12.60 (2H, br s). IR (KBr) ν 2955, 2866, 2544, 1703, 1678, 1578, 1431, 1267, 1225 cm^{-1}. EI-MS *m*/*z*: 312 (M$^+$). HRMS *m*/*z*: 312.0990 (Calcd for $C_{18}H_{16}O_5$: 312.0997). Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.14; H, 5.20.

4.3.1.3. 2-(4-Benzyloxybenzylidene)succinic acid (2c). The reaction was undertaken with *p*-benzyloxybenzaldehyde (**1c**) (5.00 g, 23.5 mmol) in dimethyl sulfoxide (30 mL) to afford **2c** (2.80 g, 38%). mp 207–208 °C (recrystallized from ethyl acetate). ^{1}H NMR (DMSO-d_6) δ 3.44 (2H, s), 5.18 (2H, s), 7.13 (2H, d, J = 8.8 Hz), 7.14–7.51 (7H, m), 7.73 (1H, s), 12.51 (2H, br s). IR (KBr) ν 2918, 2849, 2540, 1703, 1674, 1605, 1512, 1425, 1258, 1231, 1175 cm^{-1}. EI-MS *m*/*z*: 312 (M$^+$). HRMS *m*/*z*: 312.1004 (Calcd for $C_{18}H_{16}O_5$: 312.0997). Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.05; H, 5.22.

4.3.2. Procedure for the preparation of methyl esters 4a–c

Acetic anhydride (2.0 equiv) was added to a solution of acids **2a**–**c** in dichloromethane (40 mL), which was stirred for 1.5 h at 60 °C. The reaction mixture was evaporated under vacuum, and dichloromethane (6 mL) added to give a suspension. A solution of **3** (1.0 equiv) in dichloromethane (4 mL) at 0 °C was added to this suspension, and the mixture stirred for 5 h at room temperature. After the reaction, the mixture was evaporated and dissolved in methanol (20 mL). Thionyl chloride (1.5 mL) at 0 °C was added to this solution, and the mixture stirred for 15.5 h at room temperature. The reaction mixture was evaporated, poured into a saturated aqueous solution of sodium bicarbonate and extracted with ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, and evaporated under vacuum. The residue was purified using silica gel column chromatography (*n*-hexane/ethyl acetate = 1/1) to afford **4a**–**c**.

4.3.2.1. Methyl 2-(2-benzyloxybenzylidene)-4-oxo-4-(*cis*-3*a*,4,7,7*a*-tetrahydro-isoindolin-2-yl) butanoate (4a). The reaction was carried out with **2a** (2.83 g, 9.06 mmol) to afford **4a** (2.87 g, 73%). ^{1}H NMR ($CDCl_3$) δ 1.68 (2H, m), 2.23–2.32 (3H, m), 2.43 (1H, m), 3.29 (1H, dd, J = 6.9, 9.6 Hz), 3.40 (1H, m), 3.42 (2H, s), 3.53–3.61 (2H, m), 3.80 (3H, s), 5.14 (2H, s), 5.65 (2H, m), 7.27–7.42 (6H, m), 7.55 (1H, br d, J = 7.1 Hz), 8.10 (1H, s). IR ($CHCl_3$) ν 3007, 2949, 1705, 1634, 1485, 1449, 1437, 1242 cm^{-1}. FAB-MS *m*/*z*: 432 (M+H$^+$). HRMS *m*/*z*: 432.2172 (Calcd for $C_{27}H_{29}NO_4$: 432.2175).

4.3.2.2. Methyl 2-(3-benzyloxybenzylidene)-4-oxo-4-(*cis*-3*a*,4,7,7*a*-tetrahydro-isoindolin-2-yl) butanoate (4b). The reaction was undertaken with **2b** (3.00 g, 9.61 mmol) to afford **4b** (1.85 g, 45%). ^{1}H NMR ($CDCl_3$) δ 1.87–1.95 (2H, m), 2.20–2.33 (3H, m), 2.42 (1H, m), 3.28 (1H, dd, J = 6.8, 9.6 Hz), 3.37 (1H, dd, J = 5.5, 11.7 Hz), 3.41 (2H, s), 3.54 (1H, dd, J = 6.4, 11.7 Hz), 3.57 (1H, dd, J = 6.8, 9.6 Hz), 3.80 (3H, s), 5.05 (2H, s), 5.63 (2H, m), 6.94 (1H, dd, J = 2.1, 8.2 Hz), 7.02 (1H, d, J = 7.6 Hz), 7.09 (1H, br), 7.24–7.41 (6H, m), 7.85 (1H, s). IR ($CHCl_3$) ν 3007, 2951, 1709, 1636, 1437, 1242 cm^{-1}. EI-MS *m*/*z*: 431 (M$^+$). HRMS *m*/*z*: 431.2105 (Calcd for $C_{27}H_{29}NO_4$: 431.2096).

4.3.2.3. Methyl 2-(4-benzyloxybenzylidene)-4-oxo-4-(*cis*-3*a*,4,7,7*a*-tetrahydro-isoindolin-2-yl) butanoate (4c). The reaction was carried out with **2c** (3.00 g, 9.61 mmol) to afford **4c** (2.89 g, 70%). ^{1}H NMR ($CDCl_3$) δ 1.89–1.98 (2H, m), 2.23–2.36 (3H, m), 2.46 (1H, m), 3.34 (dd, 1H, J = 7.0, 9.5 Hz), 3.40 (1H, dd, J = 5.3, 11.7 Hz), 3.46 (2H, br s), 3.56 (1H, dd, J = 6.2, 11.7 Hz), 3.63 (1H, dd, J = 6.8, 9.5 Hz), 3.79 (3H, s), 5.08 (2H, s), 5.67 (2H, m), 6.97 (2H, d, J = 8.8 Hz), 7.31–7.45 (7H, m), 7.87 (1H, s). IR ($CHCl_3$) ν 3007, 2949, 1703, 1636, 1437, 1244 cm^{-1}. EI-MS *m*/*z*: 431 (M$^+$). HRMS *m*/*z*: 431.2091 (Calcd for $C_{27}H_{29}NO_4$: 431.2096).

4.3.3. Procedure for the preparation of phenol 5a–c

A catalytic amount of 10% Pd-C was added to a solution of **4a**–**c** in methanol and the mixture stirred for 10–40 h under a hydrogen atmosphere (1 atom). The mixture was filtered through Hyflo

Super-Cel® and the filtrate evaporated under vacuum. The residue was purified using silica gel column chromatography (*n*-hexane/ethyl acetate = 1/1) to afford **5a**–**c**.

4.3.3.1. Methyl 2-(2-hydroxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutanoate (5a). The reaction was undertaken with **4a** (3.05 g, 7.07 mmol) and 10% Pd-C (306.7 mg), in methanol (40 mL) for 10 h to afford **5a** (2.18 g, 89%). Two rotamers were observed (ca. 1/1). ^{1}H NMR ($CDCl_3$) δ 1.40–1.63 (8H, m), 2.21 (1H, m), 2.30 (1H, m), 2.51 (1H, m), 2.65 (1H, m), 2.95 (2H, m), 3.12 (1H, m), 3.26 (1H, m), 3.39–3.48 (3H, m), 3.646 and 3.648 (each 1.5H, s), 6.76 (1H, t, J = 7.6 Hz), 6.90 (1H, br d, J = 8.0 Hz), 7.05 (1H, br d, J = 7.6 Hz), 7.11 (1H, m), 8.94 and 8.95 (each 0.5H, s). IR ($CHCl_3$) ν 3215, 2934, 2859, 1732, 1620, 1582, 1489, 1458, 1242 cm^{-1}. EI-MS m/z: 345 (M^+). HRMS m/z: 345.1936 (Calcd for $C_{20}H_{27}NO_4$: 345.1940).

4.3.3.2. Methyl 2-(3-hydroxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutanoate (5b). The reaction was carried out with **4b** (151.9 mg, 0.35 mmol) and 10% Pd-C (15.2 mg) in methanol (4 mL) for 10 h to afford **5b** (75.5 mg, 62%). Two rotamers were observed (ca. 1/1). ^{1}H NMR ($CDCl_3$) δ 1.35–1.55 (8H, m), 2.05–2.29 (3H, m), 2.65 (2H, m), 3.11 (1.5H, m), 3.27–3.45 (4.5H, m), 3.68 and 3.69 (each 1.5H, s), 6.64 (1H, br d, J = 7.3 Hz), 6.72 (1H, m), 6.94 (1H, s), 7.09–7.14 (1H, m), 7.94 (1H, m). IR ($CHCl_3$) ν 3302, 2932, 2859, 1728, 1624, 1599, 1456, 1240 cm^{-1}. EI-MS m/z: 345 (M^+). HRMS m/z: 345.1932 (Calcd for $C_{20}H_{27}NO_4$: 345.1940).

4.3.3.3. Methyl 2-(4-hydroxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutanoate (5c). The reaction was undertaken with **4c** (161.5 mg, 0.37 mmol) and 10% Pd-C (16.2 mg), in methanol (4 mL) for 40 h to afford **5c** (127.7 mg, 99%). Two rotamers were observed (ca. 1.5/1). ^{1}H NMR ($CDCl_3$) δ 1.33–1.61 (8H, m), 2.15 (1H, m), 2.21–2.32 (2H, m), 2.66 (2H, m), 2.96 (0.6H, dd, J = 4.9, 6.4 Hz), 2.99 (0.4H, dd, J = 4.9, 6.4 Hz), 3.17 (0.6H, dd, J = 6.2, 10.1 Hz), 3.24–3.47 (4.4H, m), 3.65 (1.8H, s), 3.66 (1.2H, s), 6.75 (2H, m), 6.96–7.04 (3H, m). IR ($CHCl_3$) ν 3312, 2932, 1728, 1626, 1516, 1450, 1258, 1173 cm^{-1}. EI-MS m/z: 345 (M^+). HRMS m/z: 345.1946 (Calcd for $C_{20}H_{27}NO_4$: 345.1940).

4.3.4. Procedure for the preparation of FMIT

Step 1: Cesium carbonate (1.4 equiv) was added to a solution of **5a**–**c** and 2-fluoroethyl tosylate (1.5 equiv) in dimethyl formamide, and the mixture stirred at 60 °C. After chilling the reaction mixture to room temperature, the mixture was poured into water and extracted with diethyl ether. The organic layer was washed with a small amount of water, dried over magnesium sulfate, and evaporated under vacuum. The residue was purified using silica gel column chromatography (*n*-hexane/ethyl acetate = 1/1) to afford the methyl ester.

Step 2: An aqueous solution of sodium hydroxide (2 M) was added to a solution of the methyl ester in 1,4-dioxane, and the mixture stirred at 50 °C. After chilling the reaction mixture to room temperature, it was acidified with 1 N hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, and evaporated under vacuum. The residue was purified using silica gel column chromatography (chloroform/methanol = 9/1) to afford FMIT.

4.3.4.1. 2-(2-Fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutanoicacid (*o*-FMIT). The step-1 reaction was carried out with **5a** (386.9 mg, 1.12 mmol) in dimethyl formamide (10 mL) for 17.5 h to afford the corresponding methyl ester (386.0 mg, 88%). The step-2 reaction was undertaken with the methyl ester (386.0 mg, 0.99 mmol) and an aqueous solution of sodium hydroxide (2 M, 5 mL) in 1,4-dioxane (20 mL) for 1 h to afford *o*-FMIT (315.4 mg, 85%). Two rotamers were observed (ca. 1/1). ^{1}H NMR ($CDCl_3$) δ 1.34–1.61 (8H, m), 2.18 (2H, m), 2.53 (2H, m), 2.85 (1H, m), 2.93 (0.5H, dd, J = 5.7, 10.5 Hz), 3.06–3.28 (3.5H, m), 3.32 (0.5H, dd, J = 6.0, 12.1 Hz), 3.38 (1H, m), 3.43 (0.5H, dd, J = 7.1, 12.1 Hz), 4.22 (2H, br d, J = 27.5 Hz), 4.76 (2H, dt, J = 3.4, 47.4 Hz), 6.85 (1H, d, J = 8.2 Hz), 6.92 (1H, t, J = 7.3 Hz), 7.16 (1H, d, J = 7.6 Hz), 7.21 (1H, t, J = 7.6 Hz). IR ($CHCl_3$) ν 3007, 2934, 2859, 1732, 1587, 1495, 1450, 1242 cm^{-1}. FAB-MS m/z: 378 (M+H$^+$). HRMS m/z: 378.2076 (Calcd for $C_{21}H_{29}FNO_4$: 378.2081).

Racemic *o*-FMIT was optically resolved using preparative HPLC (acetonitrile/methanol/TFA = 95/5/0.1, 2.0 mL/min) using a chiral column. The enantiomer with a retention time of 11.6 min was (+)-*o*-FMIT, and the enantiomer with a retention time of 24.3 min was (−)-*o*-FMIT. (+)-*o*-FMIT: [α] + 6.9° (*c* 1.01, MeOH). (−)-*o*-FMIT: [α] −5.7° (*c* 0.30, MeOH).

4.3.4.2. 2-(3-Fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutanoic acid (*m*-FMIT). The step-1 reaction was carried out with **5b** (65.6 mg, 0.19 mmol) in dimethyl formamide (2 mL) for 13 h to afford the corresponding methyl ester (56.1 mg, 76%). The step-2 reaction was undertaken with the methyl ester (56.1 mg, 0.14 mmol) and an aqueous solution of sodium hydroxide (2 M, 1 mL) in 1,4-dioxane (4 mL) for 1 h to afford *m*-FMIT (37.9 mg, 72%). Two rotamers were observed (ca. 1/1). ^{1}H NMR ($CDCl_3$) δ 1.35–1.56 (8H, m), 2.20 (2H, m), 2.42–2.59 (2H, m), 2.77 (1H, m), 3.03 (0.5H, dd, J = 6.0, 10.0 Hz), 3.11–3.23 (3.5H, m), 3.33 (0.5 H, dd, J = 6.0, 12.0 Hz), 3.38 (1H, m), 3.45 (0.5H, dd, J = 6.8, 12.0 Hz), 4.20 (2H, dt, J = 4.4, 28.0 Hz), 4.74 (2H, dt, J = 4.4, 47.2 Hz), 6.78 (3H, m), 7.22 (1H, br t, J = 7.6 Hz), 12.05 (1H, br). IR ($CHCl_3$) ν 3007, 2934, 2859, 1732, 1585, 1489, 1449, 1260, 1240, 1196 cm^{-1}. FAB-MS m/z: 378 (M+H$^+$). HRMS m/z: 378.2084 (Calcd for $C_{21}H_{29}FNO_4$: 378.2081).

Racemic *m*-FMIT was optically resolved using preparative HPLC (acetonitrile/methanol/TFA = 95/5/0.1, 2.0 mL/min) using a chiral column. The enantiomer with a retention time of 12.5 min was (−)-*m*-FMIT, and the enantiomer with a retention time of 44.6 min was (+)-*m*-FMIT. (+)-*m*-FMIT: [α] + 1.8° (*c* 1.88, MeOH). (−)-*m*-FMIT: [α] −1.7° (*c* 2.52, MeOH).

4.3.4.3. 2-(4-Fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutanoic acid (*p*-FMIT). The step-1 reaction was undertaken with **5c** (57.6 mg, 0.17 mmol) in dimethyl formamide (2 mL) for 21 h to afford the corresponding methyl ester compound (48.5 mg, 73%). The step-2 reaction was carried out with the methyl ester (56.1 mg, 0.14 mmol) and an aqueous solution of sodium hydroxide (2 M, 1 mL) in 1,4-dioxane (4 mL) for 1 h to afford *p*-FMIT (15.8 mg, 35%). Two rotamers were observed (ca. 1/1). ^{1}H NMR ($CDCl_3$) δ 1.35–1.55 (8H, m), 2.18 (2H, m), 2.38 (1H, dd, J = 4.4, 16.4 Hz), 2.55 (1H, dd, J = 6.0, 16.4 Hz), 2.75 (1H, dd, J = 8.8, 13.6 Hz), 3.04–3.40 (6H, m), 4.16 (2H, dt, J = 4.0, 28.4 Hz), 4.73 (2H, dt, J = 4.0, 47.6 Hz), 6.84 (2H, d, J = 8.0 Hz), 7.12 (2H, d, J = 8.0 Hz), 11.62 (1H, br). IR ($CHCl_3$) ν 3007, 2934, 2859, 1732, 1585, 1512, 1452, 1240, 1180 cm^{-1}. EI-MS m/z: 377 (M^+). HRMS m/z: 377.2008 (Calcd for $C_{21}H_{28}FNO_4$: 377.2002).

Racemic *p*-FMIT was optically resolved using preparative HPLC (acetonitrile/methanol/TFA = 95/5/0.1, 2.0 mL/min) using a chiral column. The enantiomer with a retention time of 17.4 min was (−)-*p*-FMIT, and the enantiomer with a retention time of 28.8 min was (+)-*p*-FMIT. (+)-*p*-FMIT: [α] + 12.2° (*c* 3.72, MeOH). (−)-*p*-FMIT: [α] + 12.2° (*c* 3.74, MeOH).

4.3.5. Procedure for the preparation of MTPA esters (*S*,*R*)-6 and (*R*,*R*)-6

Isobutyl chloroformate (2.0 equiv) was added to a solution of triethylamine (2.0 equiv) and (+)-*o*-FMIT (40.8 mg, 0.11 mmol) or

(−)-*o*-FMIT (40.1 mg, 0.11 mmol) in tetrahydrofuran (1 mL), and the mixture stirred for 3 h at −10 °C. The reaction mixture was filtered, and an aqueous solution of $NaBH_4$ (5 equiv) added to the filtrate at 0 °C. The mixture was stirred for 1 h at room temperature. After the reaction the mixture was poured into a saturated aqueous solution of sodium bicarbonate and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, and evaporated under vacuum. The residue was purified using preparative silica gel TLC (chloroform/methanol = 20/1) to afford the alcohol derived from (+)-*o*-FMIT (11.7 mg, 30%) or the alcohol derived from (−)-*o*-FMIT (9.8 mg, 25%). Triethylamine (1.2 equiv), a catalytic amount of DMAP, and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (1.2 equiv) were added to a solution of the alcohol derived from (+)-*o*-FMIT (11.7 mg, 32.2 μmol) or (−)-*o*-FMIT (9.8 mg, 27.0 μmol) at 0 °C, and stirred for 5 h at room temperature. The reaction mixture was poured into a saturated aqueous solution of sodium bicarbonate and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, and evaporated under vacuum. The residue was purified using preparative silica gel TLC (*n*-hexane/ethyl acetate = 1/1) to afford (*S*,*R*)-**6** (11.3 mg, 60%) or (*R*,*R*)-**6** (11.7 mg, 75%).

4.3.5.1. (2*S*)-2-(2-Fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutyl (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetate (*S*,*R*-6). Two rotamers were observed (ca. 1/1). [α] + 23.0° (*c* 0.55, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 1.34–1.67 (8H, m), 2.20 (4H, m), 2.75 (3H, br), 3.04 (0.5H, dd, *J* = 5.7, 9.8 Hz), 3.08 (0.5H, dd, *J* = 6.0, 9.8 Hz), 3.15 (0.5H, dd, *J* = 6.9, 9.8 Hz), 3.23 (0.5 H, dd, *J* = 7.1, 9.8 Hz), 3.29–3.41 (2H, m), 3.55 and 3.56 (each 1.5H, s), 4.19 (2H, br d, *J* = 28.2 Hz), 4.27 (1H, m), 4.42–4.48 (1H, m), 4.73 (2H, dt, *J* = 3.7, 47.4 Hz), 6.82 (1H, d, *J* = 8.2 Hz), 6.88 (1H, br t, *J* = 7.3 Hz), 7.06 (1H, d, *J* = 7.3 Hz), 7.18 (1H, t, *J* = 7.8 Hz), 7.41 (3H, m), 7.53 (2H, m). IR ($CHCl_3$) *v* 3007, 2932, 2857, 1748, 1624, 1493, 1450, 1244, 1194, 1171 cm^{-1}. FAB-MS *m*/*z*: 580 ($M+H^+$). HRMS *m*/*z*: 580.2691 (Calcd for $C_{31}H_{38}F_4NO_5$: 580.2686).

The magnitude of LIS_{OMe} versus the molar ratio of Eu(fod)$_3$ for (*S*,*R*)-**6** was 0.47 ppm.

4.3.5.2. (2*R*)-2-(2-Fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutyl (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetate (*R*,*R*-6). Two rotamers were observed (ca. 1/1). [α] +23.9° (*c* 0.59, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 1.35–1.63 (8H, m), 2.16 (4H, m), 2.73 (3H, br), 3.07 (1H, m), 3.20 (1H, m), 3.31–3.43 (2H, m), 3.55 and 3.56 (each 1.5H, s), 4.20 (2H, br d, *J* = 27.9 Hz), 4.35 (2H, m), 4.74 (2H, dt, *J* = 3.9, 47.4 Hz), 6.81 (1H, d, *J* = 8.0 Hz), 6.88 (1H, t, *J* = 7.3 Hz), 7.04 (1H, d, *J* = 7.3 Hz), 7.18 (1H, t, *J* = 7.8 Hz), 7.41 (3H, m), 7.53 (2H, m). IR ($CHCl_3$) *v* 3007, 2932, 2857, 1748, 1624, 1493, 1450, 1246, 1194, 1171 cm^{-1}. FAB-MS *m*/*z*: 580 ($M+H^+$). HRMS *m*/*z*: 580.2681 (Calcd for $C_{31}H_{38}F_4NO_5$: 580.2686).

The magnitude of LIS_{OMe} versus the molar ratio of Eu(fod)$_3$ for (*R*,*R*)-**6** was 0.90 ppm.

4.3.5.3. NMR studies for determination of absolute configuration. A solution of MTPA esters **6** in $CDCl_3$ were taken with a molar ratio of Eu(fod)$_3$ to MTPA ester of 0.1–0.3 and their NMR spectra recorded. The magnitudes of the induced chemical shift of OMe signals were plotted against molar ratio (Eu(fod)$_3$/MTPA ester). In this range, the induced shifts were essentially linear with respect to the molar ratio of the reagent. The lanthanide-induced shift of **6** derived from (+)-*o*-FMIT versus molar ratio was 0.47 ppm, and that of **6** derived from (−)-*o*-FMIT was 0.90 ppm. Therefore, (+)-*o*-FMIT was determined to have an *S*-configuration, and (−)-*o*-FMIT was determined to have a *R*-configuration.

4.3.6. Methyl 2-(2-bromoethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin- 2-yl)-4-oxobutanoate (7)

Cesium carbonate (1.16 g, 3.6 mmol) was added to a solution of **5a** (560 mg, 1.6 mmol) and 2-bromoethyl *p*-toluenesulfonate (1.09 g, 3.9 mmol) in dimethyl formamide (8 mL), and the mixture stirred at 40 °C over one weekend. After chilling the reaction mixture to room temperature it was poured into water and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over magnesium sulfate, and evaporated under vacuum. The residue was purified using silica gel column chromatography (*n*-hexane/ethyl acetate = 4/1 to 1/1) to afford **7** (440 mg, 60%). Two rotamers were observed (ca. 1/1). ^{1}H NMR ($CDCl_3$) δ 1.34–1.64 (8H, m), 2.14 (1H, m), 2.22 (1H, m), 2.33 (1H, m), 2.65 (0.5H, dd, *J* = 5.7, 9.8 Hz), 2.68 (0.5H, dd, *J* = 5.3, 9.6 Hz), 2.85 (1H, m), 3.14 (1H, m), 3.17 (0.5H, dd, *J* = 6.2, 9.8 Hz), 3.26–3.42 (4.5H, m), 3.63 and 3.64 (each 1.5H, s), 3.69 (2H, br t, *J* = 4.6 Hz), 4.30 (2H, br t, *J* = 4.6 Hz), 6.81 (1H, d, *J* = 8.2 Hz), 6.90 (1H, br t, 7.3 Hz), 7.11 (1H, d, *J* = 7.6 Hz), 7.19 (1H, br t, *J* = 8.2 Hz). IR ($CHCl_3$) *v* 3007, 2932, 2859, 1728, 1628, 1493, 1454, 1248, 1194 cm^{-1}. FAB-MS *m*/*z*: 452 ($M+H^+$). HRMS *m*/*z*: 452.1434 (Calcd for $C_{22}H_{31}BrNO_4$: 452.1436).

4.4. In vitro binding assays

The displacement effect of the mitiglinide derivatives on SUR1 binding was assessed using dispersed islet cells as described previously.[27] Pancreatic islets were isolated from male ddY mice by a collagenase digestion method.[14] Isolated islets were dispersed using 0.05% trypsin/0.53 mM EDTA (Invitrogen, Carlsbad, CA, USA) and PBS. Islet cells were incubated with [^{3}H] glibenclamide (3.7 kBq) in 1 mL of buffer containing 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mg/mL bacitracin, and 1 mg/mL BSA for 1 h at room temperature in the presence of varying concentrations of the nonradioactive mitiglinide derivatives. Binding was terminated by rapid filtration through Whatman GF/C filters (24 mm) followed by washing thrice with 5 mL of ice-cold phosphate-buffered saline (PBS). The radioactivity of the filters was measured with a liquid scintillation analyzer. Results were expressed as the percentage of the radioactivity of the bound [^{3}H]glibenclamide remaining after the addition of the nonradioactive compound. GraphPad Prism software (v5.03 for Windows) was used to calculate 50% inhibitory concentration (IC_{50}) values.

4.5. Radiochemistry

4.5.1. (2*S*)-2-(2-^{18}Fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin- 2-yl)-4-oxobutanoic acid ((+)-(*S*)-*o*-[^{18}F]FMIT) and (2*R*)-2-(2-^{18}Fluoroethyloxybenzyl)-4-(*cis*-3*a*,4,5,6,7,7*a*-hexahydro-isoindolin-2-yl)-4-oxobutanoic acid ((−)-(*R*)-*o*-[^{18}F]FMIT)

[^{18}F]Fluoride trapped on an anion-exchange cartridge was eluted with an aqueous solution of potassium carbonate (33 mM). Kryptofix$_{222}$ (22.1 mg) was dissolved in the solution of [^{18}F]fluoride (9.99 GBq) in water. The solvent was removed at 120 °C under a stream of nitrogen gas. The residue was dried with anhydrous acetonitrile (0.5 mL) thrice at 120 °C under nitrogen. For (+)-(*S*)-*o*-[^{18}F]FMIT, a solution of the precursor **7** (2.83 mg) in acetonitrile (0.15 mL) was added to the reaction vessel containing the ^{18}F (4.78 MBq). The mixture was heated at 140 °C for 10 min. After the solvent was removed at room temperature under a stream of nitrogen gas, the residue was dissolved in 1,4-dioxane (0.05 mL), then an aqueous solution of sodium hydroxide (2 M, 0.05 mL) was added and the mixture stored for 1 h at room temperature. After this, acetonitrile (0.2 mL) was added and the mixture purified using HPLC with a reverse-phase column (Cosmosil 5C_{18}-AR-II,

10 × 250 mm, acetonitrile/water/TFA = 70/30/0.1) and a chiral column (CHIRALPAK AY-H, 10 × 250 mm, acetonitrile/methanol/TFA = 95/5/0.1) at a flow rate of 1.0 mL/min. Fractions containing the product were evaporated. (+)-(*S*)- and (−)-(*R*)-*o*-[^{18}F]FMIT were obtained with a radiochemical yield of 18% and 13% (overall decay corrected). The radiochemical purity of these radiotracers was >98% after purification by high-performance liquid chromatography (HPLC) and their specific activity estimated to be ≈100 GBq/μmol.

4.6. Biodistribution studies

Biodistribution studies of (+)-(*S*)- and (−)-(*R*)-*o*-[^{18}F]FMIT were conducted using six-week-old male ddY mice. *o*-[^{18}F]FMITs (0.15–0.22 MBq/100 μL) were administered to mice through tail-vein injection. At 5, 15, 30, 60, and 120 min after administration, mice were sacrificed by exsanguination. Radioactivity in blood and organs was measured using an auto well γ-counter. Radioactivity levels in tissue were expressed as a percentage of the injected dose per gram of tissue (% ID/g).

4.7. Ex vivo autoradiography experiments

(+)-(*S*)-*o*-[^{18}F]FMIT was administered to non-anesthetized ddY mice (male, weight: 20 g, $n = 3$) by intravenous injection (185 MBq/100 μL). Mice were sacrificed by exsanguination 30 min after administration. Pancreases were removed, rinsed with saline, and frozen immediately. Frozen pancreases were cut into several sections (thickness, 10 μm), placed on a glass slide, and covered with a glass cover. The radioactivity of a pancreatic section on a glass slide was determined using an imaging analyzer (BAS5000, Fujifilm Photo Film, Tokyo, Japan) (18-h exposure).

4.8. Immunohistochemistry

Serial sections of pancreas prepared for autoradiography were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h, then permeabilized and blocked with 0.5% Triton X-100 in PBS containing 1.5% goat serum for 1 h at room temperature. Specimens were incubated with rabbit anti-insulin (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a primary antibody overnight at 4 °C. After rinsing with PBS, specimens were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:200; Molecular Probes, Eugene, OR, USA) as a secondary antibody for 1 h at room temperature. Fluorescence signals were acquired through a fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan).

4.9. Statistical analyses

Data are the mean ± SD. The statistical significance of differences was evaluated using the Tukey–Kramer test. $P < 0.05$ was considered significant.

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