

Supporting Information

Synthesis and characterization of a novel ^{18}F -labeled 2,5-diarylnicotinamide derivative targeting orexin 2 receptor

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Materials and methods

General

All reagents were obtained commercially and used without further purification unless otherwise indicated. ^1H NMR spectra were obtained on a JEOL JNM400 (JEOL, Tokyo, Japan) with TMS as an internal standard. Coupling constants are reported in hertz. Multiplicity was defined by s (singlet), d (doublet), and m (multiplet). Mass spectra were obtained on a SHIMADZU LCMS-2020 EV. High-resolution mass spectrometry (HRMS) was carried out with a JEOL JMS-700 (JEOL). Reversed-phase high-performance liquid chromatography (HPLC) was performed with a Shimadzu system (LC-20AD pump with SPD-20A UV detector, $\lambda = 254$ nm) using a Cosmosil C18 column (5C₁₈-AR-II 4.6 mm I.D.×150 mm, Nacalai Tesque, Kyoto, Japan). Animal experiments were conducted in accordance with our institutional guidelines and were approved by Kyoto University.

Chemistry

Methyl 6-chloro-[3,3'-bipyridine]-5-carboxylate (1).

A mixture of methyl 5-bromo-2-chloronicotinate (250 mg, 1 mmol), pyridine-3-ylboronic acid (61 mg, 0.5 mmol), Cs₂CO₃ (650 mg, 4 mmol), and PdCl₂(dppf) (86

mg, 0.1 mmol) in DMF (10.8 mL)/H₂O (360 μ L) was stirred at 160 °C for 20 min under argon gas. After cooling to room temperature, the reaction solution was added with H₂O (50 mL) and extracted with ethyl acetate (80 mL). The extracted solution was washed with brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (chloroform/methanol = 19/1) to give **1** as a black solid (184 mg, 74%). ¹H NMR (400 MHz, CDCl₃) δ 8.86 (dd, J = 2.0 Hz, 1H), 8.74 (d, J = 2.4 Hz, 1H), 8.72 (dd, J = 3.2 Hz, 1H), 8.38 (d, J = 2.4 Hz, 1H), 7.92-7.89 (m, 1H), 7.48-7.44 (m, 1H), 4.01 (s, 3H). MS (ESI) m/z 249.0 [MH]⁺.

Methyl 6-phenyl-[3,3'-bipyridine]-5-carboxylate (2).

A mixture of **1** (680 mg, 2.7 mmol), phenylboronic acid (402 mg, 3.3 mmol), Cs₂CO₃ (3518 mg, 10.8 mmol), and PdCl₂(dppf) (220 mg, 0.27 mmol) in DMF (27.8 mL)/H₂O (1 mL) was stirred at 100 °C for 3 h under argon gas. After cooling to room temperature, the reaction solution was added with H₂O (50 mL) and extracted with ethyl acetate (80 mL). The extracted solution was washed with brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (ethyl acetate/hexane = 1/2) to give **2** as a brown solid (190 mg, 24%). ¹H NMR (400 MHz, CDCl₃) δ 9.00 (d, J = 2.4 Hz, 1H), 8.96 (d, J = 2.4 Hz, 1H), 8.74

(dd, $J = 5.2$ Hz, 1H), 8.30 (dd, $J = 2.4$ Hz, 1H), 8.00-7.97 (m, 1H), 7.61-7.58 (m, 2H), 7.49-7.46 (m, 4H), 3.75 (s, 3H). MS (ESI) m/z 291.1 [MH]⁺.

N-(4-Hydroxy-3-methoxybenzyl)-6-phenyl-[3,3'-bipyridine]-5-carboxamide (**3**).

A solution of **2** (190 mg, 0.66 mmol) in THF (2.5 mL)/methanol (2.5 mL) was added with 1N KOH aqueous solution (1 mL), and the reaction solution was stirred at room temperature for 2 days under argon gas. The reaction solution was azeotroped with ethyl acetate (10 mL). To a solution of the residue in DMF (3.2 mL) were added 3-(aminomethyl)-5-methoxyphenol hydrochloride (148 mg, 0.8 mmol) dissolved in 5 N NaOH (310 μ L) and H₂O (1.8 mL), DIPEA (340 mg, 2.6 mmol), EDC · HCl (503 mg, 2.6 mmol), and HOAt (357 mg, 2.6 mmol). This was stirred at 100 °C for 3 h under argon gas. After cooling down to room temperature, the reaction solution was neutralized with 2 N HCl, H₂O (50 mL) and then extracted with ethyl acetate (80 mL). The extracted solution was washed with NaHCO₃ saturated water, dried over magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (chloroform/methanol = 19/1) to give **3** as a pink solid (71 mg, 26%). ¹H NMR (400 MHz, CDCl₃) δ 8.98-8.97 (m, 1H), 8.93 (d, $J = 2.4$ Hz, 1H), 8.74-8.71 (m, 1H), 8.26 (d, $J = 2.8$ Hz, 1H), 7.98-7.96 (m, 1H), 7.74-7.69 (m, 2H), 7.49-7.45

(m, 4H), 6.86 (s, 1H), 6.84 (s, 1H), 6.76 (s, 1H), 5.58 (s, 1H), 5.31 (s, 1H), 4.30 (d, $J = 3.0$ Hz, 2H), 3.86 (s, 3H). MS (APCI) m/z 412.2 [MH]⁺.

N-(4-(2-Fluoroethoxy)-3-methoxybenzyl)-6-phenyl-[3,3'-bipyridine]-5-carboxamide (**4**, **DAN-1**).

A mixture of **3** (81 mg, 0.2 mmol), 2-fluoroethyl 4-methylbenzenesulfonate (65 mg, 0.3 mmol), and K₂CO₃ (55 mg, 0.4 mmol) in DMF (5 mL) was stirred at 80 °C for 3 h under argon gas. After cooling down to room temperature, the reaction solution was added with H₂O (30 mL) and extracted with ethyl acetate (80 mL). The extracted solution was washed twice with brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (chloroform/methanol = 19/1) to give **4** as a white solid (38 mg, 43%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.10 (d, $J = 2.4$ Hz, 1H), 9.07 (d, $J = 2.4$ Hz, 1H), 9.02 (t, $J = 5.8$ Hz, 1H), 8.66 (d, $J = 4.0$ Hz, 1H), 8.27 (d, $J = 8.0$ Hz, 1H), 8.23 (d, $J = 1.6$ Hz, 1H), 7.66 (d, $J = 6.8$ Hz, 2H), 7.62-7.49 (m, 2H), 7.43-7.34 (m, 3H), 6.91-6.89 (m, 2H), 6.72 (dd, $J = 9.6$ Hz, 1H), 4.79 (dt, $J = 7.6$ Hz, 1H), 4.67 (dt, $J = 7.6$ Hz, 1H), 4.33 (d, $J = 5.6$ Hz, 2H), 4.16 (dt, $J = 7.6$ Hz, 1H), 3.72 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 167.7, 155.4, 149.8, 149.7, 148.8, 148.1, 147.5, 138.2, 135.7, 134.4, 131.9, 131.2, 130.6, 129.4, 129.0,

128.9, 128.7, 123.9, 120.3, 114.3, 112.0, 81.9 (d, $J_F = 173.5$ Hz), 68.5 (d, $J_F = 22.9$ Hz), 56.0, 44.4. HRMS (FAB) m/z calcd for $C_{27}H_{25}FN_3O_3$ (MH^+) 458.1802, found 458.1875.

2-(2-Methoxy-4-((6-phenyl-[3,3'-bipyridine]-5-carboxamido)methyl)phenoxy)ethyl 4-methylbenzenesulfonate (5).

A mixture of **3** (93 mg, 0.2 mmol), ethane-1,2-diyl bis(4-methylbenzenesulfonate) (167 mg, 0.4 mmol), and K_2CO_3 (62 mg, 0.4 mmol) in DMF (5.4 mL) was stirred at 80 °C for 3 h under argon gas. After cooling down to room temperature, the reaction solution was added with H_2O (50 mL) and extracted with ethyl acetate (80 mL). The extracted solution was washed twice with brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (chloroform/methanol = 19/1) to give **5** as an orange solid (20 mg, 15%). 1H NMR (400 MHz, $CDCl_3$) δ 9.14 (s, 1H), 9.02 (s, 1H), 8.83 (s, 1H), 8.45 (d, $J = 3.8$ Hz, 1H), 8.33 (s, 1H), 7.81 (d, $J = 4.2$ Hz, 2H), 7.66 (d, $J = 3.4$ Hz, 2H), 7.45-7.41 (m, 3H), 6.82 (s, 1H), 6.77 (s, 1H), 6.71 (d, $J = 4.0$ Hz, 1H), 6.63 (s, 1H), 6.51 (d, $J = 3.8$ Hz, 1H), 6.15 (s, 1H), 4.36-4.34 (m, 3H), 4.21-4.19 (m, 3H), 3.76 (s, 3H), 2.45 (s, 3H), 2.07 (s, 1H). MS (ESI) m/z 610.2 [MH] $^+$.

***In vitro* competitive inhibition assay**

Chinese hamster ovary (CHO)-K1 cells stably expressing human OX₁R (CHO-OX₁R) or OX₂R (CHO-OX₂R) were seeded in a 12-well-plate (5×10^5 cells per well) and then incubated with 10% fetal bovine serum (FBS)/ Ham's F 12 medium at 37 °C for 24 h. After the incubation, cells were treated with 500 µL of various concentrations of DAN-1 and 500 µL of [¹²⁵I]orexin A solution (1.8 kBq/mL, PerkinElmer, Waltham, MA) at 37 °C for 2 h. The cells were washed once with 500 µL of Ham's F 12 medium and then dissolved with 1 mL of 1 N NaOH. The cell lysis solutions were poured into tubes. The radioactivity (counts per minute, CPM) of each tube was measured using an automatic γ -counter (Wallac WIZARD 2470, PerkinElmer). The IC₅₀ values of compound to orexin A were calculated using GraphPad Prism 6.0.

Radiosynthesis

An aqueous solution of [¹⁸F]KF was added to a reaction vessel containing Kryptofix 2.2.2 (8-10 mg) and anhydrous MeCN (900 µL). The mixture was dried at 120 °C under a stream of argon gas. The drying procedure was repeated three times until the mixture was completely dry. To prepare [¹⁸F]DAN-1, a solution of the tosyl precursor (**5**) in MeCN (300 µL) was added to the reaction vessel and heated at 100 °C

for 10 min. After the reaction, the mixture was purified with HPLC (MeCN/H₂O = 23/77 to 100/0 over 60 min) using a Cosmosil C₁₈ column (5C₁₈-AR-II, 4.6 × 150 mm, Nacalai Tesque).

Cellular binding study

CHO-K1, CHO-OX₁R or CHO-OX₂R cells were seeded in a 6-well-plate (1 × 10⁶ cells per well) and then incubated with 10% FBS/ Ham's F 12 medium at 37 °C for 24 h. After incubation, the cells were treated with 2 mL of [¹⁸F]DAN-1 (17 kBq) at room temperature for 1.5 h. The cells were washed with 1 mL of 0.1% BSA, 1% DMSO/PBS (×3), and dissolved with 1 mL of 1 N NaOH. The cell lysis solutions were poured into tubes. The radioactivity of each tube was measured using an automatic γ-counter. For blocking, cells were treated with 1 mL of the ¹⁸F-labeled compound (8 kBq) and 1 mL of almorexant (dual OXR antagonist¹) solution (5 μM) at room temperature for 1.5 h.

***In vitro* autoradiography**

Mouse brain sections (20 μM thick) were used in this study. The section was incubated with a 10% EtOH solution of [¹⁸F]DAN-1 (370 kBq/mL) at room temperature

for 2 h. For blocking experiments, adjacent sections were incubated with a 10% EtOH solution of [^{18}F]DAN-1 (370 kBq/mL) in the presence of almorexant (10 μM). The sections were washed in 50% EtOH for 1 hr. After drying, the sections were exposed to a BAS imaging plate (Fuji Film, Tokyo, Japan) for 1 h. Autoradiographic images were obtained using a BAS5000 scanner system (Fuji Film).

Biodistribution

The biodistribution study was conducted using five-week-old male ddY mice. [^{18}F]DAN-1 (37 kBq/100 μL in 10% EtOH) was administered to mice by tail-vein injection. At 2, 10, 30, and 60 min after administration, mice were sacrificed by decapitation. Radioactivity in blood and organs was measured using an automatic γ -counter. Radioactivity levels in tissue were expressed as a percentage of the injected dose per gram of tissue (% ID/g).

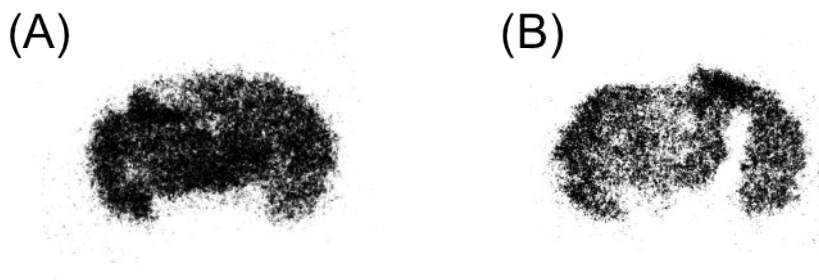


Figure S1. *In vitro* autoradiograms of [¹⁸F]DAN-1 (A) and [¹⁸F]DAN-1 + almorexant (10 μM) (B).

***In vivo* blocking study**

[¹⁸F]DAN-1 (70 kBq/100 μL of 10% EtOH supplemented with 2% DMSO and 0.5% Tween 80) was injected to five-week-old male ddY mice by tail-vein injection. For *in vivo* blocking studies, DAN-1, almorexant, or TCS1102 (1 mg/kg) was co-injected to the mice via tail vein injection. At 30 min after injection, mice were sacrificed by decapitation. Radioactivity in the brain and blood was measured using an automatic γ-counter, and expressed as a percentage of the injected dose per gram (% ID/g).

Table S1. Biodistribution of radioactivity after intravenous injection of [¹⁸F]DAN-1 in normal mice at 30 min after injection^a

Tissue	[¹⁸ F]DAN-1	[¹⁸ F]DAN-1 + DAN-1	[¹⁸ F]DAN-1 + almorexant	[¹⁸ F]DAN-1 + TCS1102
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Brain	1.94 (0.21)	1.58 (0.19)	1.72 (0.17)	1.68 (0.14)
Blood	2.77 (0.24)	2.48 (0.22)	2.59 (0.28)	2.55 (0.36)
Brain/Blood	0.70	0.63	0.66	0.65

^aExpressed as % injected dose per gram. Each value represents the mean (SD) for 5 animals.

Statistical analysis

The significance of differences was assessed by Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were regarded as significant.