Supporting Information

^{18}F-Labeled pyrido[3,4-*d*]pyrimidine as an effective probe for imaging of L858R-mutant epidermal growth factor receptor

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General

All reagents were obtained commercially and used without further purification. Mass spectra were measured using a JMS-700 MStation mass spectrometer (JEOL, Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^{1}H-NMR and at 100 MHz for ^{13}C-NMR using JNM-ECP400 (JEOL), and chemical shifts were expressed as parts per million downfield from tetramethylsilane, which served as an internal standard (δ value). The *J* values are given in Hz and are defined as singlet (s), doublet (d), triplet (t), multiplet (m), and broad (br). Dry thermos bath (MG-2200, EYELA, Tokyo) customized was used for heating in the radiosynthesis. A LC-20AD system (Shimadzu, Kyoto, Japan) was used for purification and analysis by high performance liquid chromatography (HPLC) equipped with a SPD-20A ultraviolet detector (Shimadzu) and an US-3000T radioisotope detector (Universal Giken, Odawara, Japan) and a Cosmosil 5C_{18}-AR-II (10× 250 mm, Nacalai Tesque, Kyoto, Japan) as a reversed-phase HPLC column.

Synthesis

***(2Z)-N*-[4-(3-Bromoanilino)pyrido[3,4-*d*]pyrimidin-6-yl]-4-[(4-*tert*-butoxycarbonyl) piperazin-1-yl]2-butenamide (2)**

3-Bromocrotonic acid (105.6 mg, 0.64 mmol) was added to a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (122.7 mg, 0.64 mmol) in DMF (113 μL) at 0°C, and the mixture was stirred for 1 h at 0°C. Next, a solution of 6-amino-4-(3-bromoanilino) pyrido[3,4-*d*]pyrimidine (**1**) (25.3 mg, 0.08 mmol) in DMF (346 μL) and trimethylamine (109 μL, 0.64 mmol) was added to the mixture, which was then stirred for 1 h. Finally, 1-(*tert*-butyloxylcarbonyl)piperazine (298.0 mg, 1.60 mmol) was added to the reaction mixture, and the mixture was further stirred for 30 min. The reaction was quenched by using ice water, and the mixture was poured into a 2% sodium carbonate aqueous solution and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium carbonate, and evaporated under vacuum. The residue was purified by preparative silica-gel thin-layer chromatography (chloroform : methanol = 10 : 1) to obtain compound **2** (41.4 mg, 91%) as a light brown solid. ^{1}H-NMR (400 MHz, DMSO-d_6) δ : 10.91 (1H, s), 10.26 (1H, br), 9.02 (1H, s),

8.98 (1H, s), 8.64 (1H, s), 8.16 (1H, br s), 7.89 (1H, br d, *J* = 7.7 Hz), 7.39–7.32 (2H, m), 6.87 (1H, dt, *J* = 15.4, 5.8 Hz), 6.54 (1H, d, *J* = 15.4 Hz), 3.18 (2H, d, *J* = 5.8 Hz), 2.50 (4H, m), 2.37 (4H, br t, *J* = 4.8 Hz), 1.40 (9H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 163.8, 156.6, 154.9, 154.7, 153.6, 151.9, 147.3, 143.8, 143.2, 142.3, 139.2, 130.2, 127.7, 125.3, 124.8, 122.5, 121.0, 120.3, 101.5, 79.8, 59.1, 53.1 (2C), 43.6 (2C), 28.4 (3C); IR ($CHCl_3$): 3408, 3304, 1684, 1597, 1518, 1248, 1169, 1130 cm^{-1}; MS (EI^+) *m/z*: 569 ($[M+2]^+$, 18), 567 (M^+, 18), 385 (72), 383 (100), 354 (18), 352 (12), 316 (12), 314 (10), 57 (33); HRMS (EI^+) *m/z*: 567.1597 ($C_{26}H_{30}BrN_7O_3$: 567.1593).

***(2Z)-N*-[4-(3-Bromoanilino)pyrido[3,4-*d*]pyrimidin-6-yl]-4-(piperazin-1-yl)2-buten amide (3)**

Trifluoroacetic acid (260 μL) was added to a solution of compound **2** (11.4 mg, 0.02 mmol) in chloroform (520 μL) and the mixture was stirred for 30 min at room temperature. After the reaction, the solution was neutralized by mixing with a saturated aqueous solution of sodium bicarbonate and the mixture was extracted with chloroform. The organic layer was dried over sodium sulfate and concentrated under vacuum. The residue was purified by preparative amino silica-gel thin-layer chromatography (chloroform : methanol = 20 : 1) to give compound **3** (8.0 mg, 85%) as a pale-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.90 (1H, s), 10.28 (1H, br), 9.01 (1H, s), 8.98 (1H, s), 8.63 (1H, s), 8.15 (1H, s), 7.87 (1H, br d, *J* = 7.4 Hz), 7.37 (1H, t, *J* = 8.1 Hz), 7.35–7.32 (1H, m), 6.87 (1H, dt, *J* = 15.4, 5.8 Hz), 6.53 (1H, d, *J* = 15.4 Hz), 3.12 (2H, br d, *J* = 5.8 Hz), 2.72 (4H, t, *J* = 4.8 Hz), 2.33 (4H, br); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 163.4, 156.7, 153.9, 150.7, 148.1, 142.2, 141.5, 140.7, 130.3, 126.5, 125.5, 124.8, 121.3, 121.2, 121.0, 103.4, 59.2, 54.1 (2C), 45.5 (2C); IR ($CHCl_3$): 3416, 3273, 1682, 1560, 1533 cm^{-1}; MS (EI^+) *m/z*: 469 ($[M+2]^+$, 64), 467 (M^+, 64), 384 (95), 382 (100), 316 (29), 314 (23), 153 (85), 125 (63); HRMS (EI^+) *m/z*: 467.1064 ($C_{21}H_{22}BrN_7O$: 467.1069).

***(E)-N*-[4-(3-bromoanilino)pyrido[3,4-*d*]pyrimidin-6-yl]-4-[4-(2-fluoroethyl)piperazin-1-yl]but-2-enamide (APP-1)**

Triethylamine (11.7 μL, 0.084 mmol) and 2-fluoroethyl tosylate (12.4 mg, 0.057 mmol)

were added to a solution of compound **3** (12.7 mg, 0.027 mmol) in DMF (1 mL), and the mixture was stirred for 3 h at 90°C. After the reaction, the reaction mixture was poured into a saturated aqueous solution of sodium bicarbonate, and the mixture was extracted by using chloroform. The organic layer was dried over sodium sulfate and concentrated under vacuum. The residue was purified by preparative amino silica-gel thin-layer chromatography (chloroform : methanol = 20 : 1) to give APP-1 (6.3 mg, 45%) as a pale-yellow amorphous solid. ^{1}H-NMR (400 MHz, DMSO-d_6) δ:10.91 (1H, s), 10.27 (1H, br s), 9.02 (1H, s), 8.99 (1H, s), 8.64 (1H, br s), 8.16 (1H, br s), 7.89 (1H, br d, J = 7.4 Hz), 7.34–7.37 (2H, m), 6.86 (1H, dt, J = 5.9, 15.4 Hz), 6.53 (1H, d, J = 15.4 Hz), 4.52 (2H, dt, J = 4.4, 48.0 Hz), 3.15 (2H, d, J = 5.9 Hz), 2.61 (2H, dt, J = 4.4, 28.6 Hz), 2.44 (8H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 163.8, 156.5, 154.9, 152.2, 147.5, 144.2, 142.4, 139.1, 130.3, 127.9, 125.2, 124.8, 122.7, 120.9, 120.2, 101.0, 81.9 (d, J_{CF} = 168 Hz), 59.1, 58.2 (d, J_{CCF} = 20.0 Hz), 53.5 (2C), 53.3 (2C); IR ($CHCl_3$): 3410, 3296, 1684, 1597, 1516 cm^{-1}; MS (EI$^+$) *m/z*: 515 ([M+2]$^+$, 5), 513 (M$^+$, 5), 495 (3), 493 (3), 432 (15), 383 (100), 381 (96), 354 (40), 352 (38); HRMS (EI$^+$) *m/z*: 513.1286 ($C_{23}H_{25}BrFN_7O$: 513.1288).

Epidermal growth factor receptor (EGFR) inhibition assay

The inhibition of EGFR was examined by using an EGFR Kinase Enzyme System (Promega, Catalog #: V1991) and a ADP-Glo^{TM} Kinase Assay Kit (Promega, Catalog #: V9101), which quantify the amount of adenosine diphosphate (ADP) produced from the kinase reaction by depleting the remaining adenosine triphosphate (ATP), followed by regeneration of the consumed ADP to ATP. The amount of regenerated ATP is measured based on the luminescence signal from luciferin. The experiment was done according to the Kinase Enzyme System protocol. In brief, inhibitor compounds in various concentrations were combined with recombinant EGFR kinase (30 ng) diluted in a reaction buffer (40 mM Tris–HCl, pH = 7.5, 20 mM $MgCl_2$, 50 μM DTT, and 0.1 mg/mL BSA; bovine serum albumin). After incubation for 10 min at room temperature, ATP (10 μM) was added to start the kinase reaction. The reaction was done at room temperature for 2 h in a total volume of 10 μL (2 μL of inhibitors, 4 μL of EGFR kinase solution, and 4 μL of ATP). To stop the reaction, 10 μL of ADP-Glo Reagent was added

and incubated at room temperature for 40 min, following which 20 μL of Kinase Detection Reagent was added for a further 1 h of incubation. Luminescence was measured by using a plate reader (ARVO, Perkin Elmer), and IC_{50} values were calculated from the inhibition curve by using the GraphPad Prism software (GraphPad Software, San Diego, USA).

Radiolabeling

2-[^{18}F]Fluoroethyl tosylate

Aqueous solution of potassium [^{18}F]fluoride (2.22−4.51 GBq) was added to a reaction vessel (brown glass vial) containing Kryptofix 2.2.2 (4–5 mg) and anhydrous MeCN (500 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. A solution of ethylenglycol-1,2-ditosylete (2–3 mg) in anhydrous MeCN (250 μL) was added to the dried mixture, which was heated at 90°C for 5 min. After the reaction, the mixture was diluted with water (150 μL) and purified by HPLC (acetonitrile/water/TFA = 50/50/0.01, flow rate = 4.0 mL/min), and the eluate was collected after 10–11 min. The eluate was diluted with 6–8 times its volume of water, and loaded into a Sep-Pak C18 Light Cartridge (Nihon Waters, Tokyo, Japan). The cartridge was washed with water to remove the TFA and dried with argon gas. Elution with anhydrous DMF (100 μL) gave a solution of 2-[^{18}F]fluoroethyl tosylate (0.54–1.33 GBq, n = 5).

***(E)-N*-[4-(3-bromoanilino)pyrido[3,4-*d*]pyrimidin-6-yl]-4-[4-(2-[^{18}F]fluoroethyl)piperazin-1-yl]but-2-enamide ([^{18}F]APP-1)**

A mixture of compound **3** (0.5 mg, 1.1 μmol) and Et_3N (1.45 μL) in anhydrous DMF (20 μL) was added to a solution of 2-[^{18}F]fluoroethyl tosylate (0.54–1.33 GBq) in anhydrous DMF (100 μL) in a reaction vessel, and the mixture was heated at 110°C in a sealed vial for 20 min. After the reaction, the mixture was purified by HPLC (acetonitrile/water/TFA = 80/20/0.01 at 0 min to 60/40/0.01 at 20 min (gradient), flow rate = 5.0 mL/min, λ = 280 nm), and the eluate was collected after 11.0–11.5 min to give a solution of [^{18}F]APP-1 (43.7–165 MBq, n = 5) with radiochemical yield of 3.2 ± 0.94% (end of synthesis, from potassium [^{18}F]fluoride). The isolated [^{18}F]APP-1 was

identified by a HPLC analysis with co-injection of APP-1. The radiochemical purity and the specific activity exceeded 95% and 40.4 GBq/μmol, respectively.

The solution was diluted with 20 mL of water, and loaded into a Sep-Pak C18 Light Cartridge for evaluation of [^{18}F] APP-1. The cartridge was washed with water to remove the TFA; subsequently, the product was eluted with MeCN, dried by evaporation, and dissolved in saline containing 0.5% Tween80.

Cell culture

The cells were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (Nacalai Tesque, Kyoto, Japan) supplemented with fetal bovine serum (FBS; 20% for H3255 and 10% for H1975), 100 U/mL penicillin, 100 g/mL streptomycin, and incubated at 37°C in a well-humidified incubator with 5% CO_2 and 95% ambient air.

Cell-uptake study

The H3255 and H1975 cell lines were cultured in 12-well plates. Cells were incubated in FBS-free DMEM/Ham's F-12 medium with and without AZD9291 (0, 1, 5 μM) for 30 min. Subsequently, [^{18}F]APP-1 (0.15 MBq) was added to each well and incubated for 120 min and then washed three times with PBS (-)/1% DMSO/0.1% Tween 80. Sodium hydroxide (0.2 M) was added to lyse the cells, and the radioactivity was measured with a γ counter (WALLAC Wizard, PerkinElmer, Finland). The protein concentration was measured by using the BCA Protein Assay Kit (Pierce, Rockford, USA) and used for normalization.

Animals

Animal studies were conducted in accordance with our institutional guidelines, and the experimental procedures were approved by the Kyoto University Animal Care Committee. Five-week-old male Balb/c nude mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Tumor-model mice were prepared by subcutaneous injection of cells (1.0×10^7 H3255 cells in the right shoulder; 3.0×10^6 H1975 cells in the left shoulder) suspended in 100 μL Matrigel™ (BD Biosciences, San Jose, USA).

Approximately 4 weeks after implantation, the mice underwent tracer studies.

***In vivo* biodistribution study**

[^{18}F]APP-1 (0.22 MBq) in saline/0.5% Tween was injected intravenously into the tails of H3255 tumor-bearing nude mice. The mice were sacrificed at 1 or 3 h post-injection. Tumors and organs of interest were removed and weighed, and the radioactivity was measured by using a γ counter (WALLAC Wizard, PerkinElmer, Finland). A blocking experiment was done by co-injection of [^{18}F]APP-1 and an excess of AZD9291 (17 μg in saline containing 5% DMSO and 0.5% Tween) and the mice were sacrificed at 3 h post-injection.

PET imaging

PET scans were acquired by using a Triumph (TriFoil Imaging Inc., Chatsworth, CA, USA) designed for laboratory animals. [^{18}F]APP-1 (13 MBq) was injected intravenously into the tails of H3255 or H1975 tumor-bearing nude mice and PET scanning was started 3 h post-injection. Dynamic PET images were acquired for 20 min. The images were reconstructed using three-dimensional ordered subset expectation maximization (3D-OSEM). After PET imaging, we removed and weighed tumors, blood, muscle, and lungs, and measured the radioactivity by using the γ counter.

Statistical analysis

The statistical significance of any difference between groups was determined by applying a Student's t-tests. $P < 0.05$ was considered statistically significant.