

Supporting information

Discovery of [^{18}F]N-(2-(diethylamino)ethyl)-6-fluoronicotinamide: A melanoma positron emission tomography imaging radiotracer with high tumor to body contrast ratio and rapid renal clearance.

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I. Chemistry supporting information

a. General chemistry methods

All commercially available chemicals were purchased from Sigma-Aldrich or Lancaster, and used without further purification. [^{18}F]HF was produced by Cyclotek, Melbourne, Australia via the $^{18}\text{O}(\text{p}, \text{n})$ reaction. NMR spectroscopy was performed on a Bruker Avance DPX 400 operating at 400 MHz for ^1H NMR spectra and 100 MHz for ^{13}C NMR spectra. Low resolution mass spectrometry (LRMS) was performed on a Micromass ZMD quadrupole mass spectrometer. Fragment ions were observed for several compounds and the masses are reported. However, the identity of these fragments was not determined. Preparative HPLC was performed on a Waters Empower system, equipped with a Waters 600 gradient pump and a 996 PDA detector, 717 autosampler and fraction collector III. Purification of the radiotracers was performed on a radio-HPLC system equipped with Waters 486 UV, set at 254 nm and an Ortec Acemate Scint 925 γ -detector with a sodium iodide crystal. For radiochemical purity and stability measurements, the radiofluorinated-tracer was ascertained on an analytical HPLC system equipped with a Varian 9002 pump, linear UV detector set at 254 nm and an Ortec Acemate Scint 925 γ -detector with a sodium iodide crystal using Laura version 3 software package. The radiochemical and chemical purity and specific activity measurements were confirmed on an analytical HPLC using a C18 analytical column. Metabolite studies were performed on a system

consisting of a Waters 600 gradient pump and 510 pump, a 486 UV detector set at 254 nm, and an in line detector for measurement of radioactivity (β -Ram IN/US) operated by Waters Empower software.

b. Radiochemistry methods and results

An aqueous [^{18}F]fluoride solution (15 - 25 GBq) was added to a 10 mL vial containing anhydrous acetonitrile (1 mL), $\text{K}_{2.2.2}$ (1 equiv) and K_2CO_3 (1 equiv). The solvent was evaporated under a stream of nitrogen at 100 °C under vacuum to produce the $\text{K}[^{18}\text{F}]\text{-K}_{2.2.2}$ complex. This azeotropic drying was repeated twice by further addition of anhydrous acetonitrile (2×1 mL). The chloro-precursor (**1**, **3**, **5** or **7**) (5 mg) was dissolved in anhydrous DMF (1 mL) and added to the dried $\text{K}[^{18}\text{F}]\text{-K}_{2.2.2}$ complex. The reaction was stirred and heated at 150 °C for 5 min before an aliquot of the reaction mixture (100 μL) was diluted with mobile phase (500 μL) and purified by semi-preparative reverse phase chromatography (Table 1). The collected radioactive peak was evaporated *in vacuo* and formulated to a concentration of 1 MBq/100 μL of saline containing less than 1% ethanol for biological studies.

Table 1. Radiolabelling data for the [^{18}F]fluoronicotinamides.

[^{18}F]Cmpd	Purification Solvent ^a	Flow rate ^b	Retention time (min)	RCY % ^c
[^{18}F] 2	20/80	2.5 mL/min	19	35-45
[^{18}F] 4	30/70	3 mL/min	15	45-55
[^{18}F] 6	20/80	3 mL/min	16	40-55
[^{18}F] 8	20/80	3 mL/min	16	30-40

^a Acetonitrile/ammonium bicarbonate solution 20 mM pH 8.

^b Phenomenex bondclone C18 (10 μm , 7.8×300 mm).

^c Isolated yield (not decay corrected), specific activity 150-220 GBq/ μmol .

c. Lipophilicity

The lipophilicity of **2**, **4**, **6** and **8** was assessed using RP-HPLC by determining the log $P_{7.5}$ value using literature procedures.¹ Compounds **2**, **4**, **6**, or **8** were dissolved in methanol, were analysed using a Waters, Xterra C18 column (5 μ m, 4.6 \times 150 mm) with a mobile phase consisting of methanol/phosphate buffer (0.1 M, pH 7.5), 60/40 and a flow rate of 1 mL/min. The log $P_{7.5}$ of a studied compound was estimated by a comparison of its retention time to that of compounds of known log P value (aniline, benzene, bromobenzene and ethylbenzene) (Table 2).

Table 2. Lipophilicity, log $P_{7.5}$ for the fluoronicotinamide compounds

Compound	log $P_{7.5}$
2	1.10
4	1.52
6	0.94
8	1.22

d. Metabolite studies

The determination of unchanged radiotracer in the plasma, tumor, eye and urine was performed by radio HPLC analysis at 0.25, 0.5, 1 and 2 h after injection of 18.5 MBq of the [¹⁸F]nicotinamide compounds. Plasma (50 μ L) was mixed with water (200 μ L) containing the fluoronicotinamide compound (10 nmol in 10 μ L methanol) and KF (200 nmol) for direct injection onto the HPLC. Organ tissue samples (15-50 mg) in 0.5 mL acetonitrile/water (3:2) containing unlabelled fluoronicotinamide and KF was exposed for 2 min to an ultrasonic probe and the mixture centrifuged at 3000 rpm for 5 min. The supernatant was collected and 100 μ L of this solution was diluted with 1.5 mL of H₂O for HPLC analysis. The radioactivity of the precipitate was measured to quantify the acetonitrile/water extraction efficiency.

Radio-HPLC analysis was performed by literature procedure² using a precolumn (Waters Oasis HLB, 25 μ m; 3.9 \times 20 mm) and a reverse phase HPLC column (Phenomenex C18 bondclone, 10 μ m; 4.6 \times 250mm) in series, with a switching valve between columns. The precolumn was used to wash protein residue while trapping all non-polar activity for subsequent gradient separation using the HPLC column. In the first step, the precolumn was washed with 1% acetonitrile in water for 3 min at 1.5 mL/min and the fraction containing protein residue and non lipophilic compounds bypassed the HPLC column and sent directly through the UV and radioactivity detectors. In the second step, the solvent direction was changed at 3 min to the HPLC column. Both columns in series were then eluted with 40/60 acetonitrile/ammonium bicarbonate pH 8.0, 20 mM over 10 min at 1.5 mL/min. The radioactivity peak corresponding to the authentic fluoronicotinamide was compared to the total activity registered in the radiochromatogram to give the fraction of unchanged ligand in the sample.

e. Synthesis and characterisation

General procedure A for preparation of fluoro and chloro - nicotinamide and isonicotinamide derivatives

To a solution of the halo-nicotinic acid or halo-isonicotinic acid (1-6 mmol) in dimethyl formamide (DMF) (5 mL) was added the appropriate amine (1.1 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (1.1 equiv), 1-hydroxybenzotriazole hydrate (HOBT) (1.1 equiv) and *N,N*-diisopropylethyl amine (DIPEA) (2.0 equiv). The reaction was allowed to stir at room temperature for 20 h before it was diluted with water (10 mL) and then extracted into DCM (2 \times 20 mL)

and the combined organics washed with dilute NaHCO₃ solution (4 × 20 mL), dried over MgSO₄, filtered and evaporated.

Synthesis of nicotinamides for [¹⁸F] labelling (1-8)

N-(2-(diethylamino)ethyl)-6-chloronicotinamide (1)

General procedure A was applied to 6-chloronicotinic acid (400 mg, 2.53 mmol) and *N,N*-diethylethylenediamine followed by column chromatography with EtOAc:MeOH:NH₃ (10:1:0.1) to give the title compound as a clear oil (514 mg, 79.1 %). ¹H NMR (CDCl₃, 400 MHz) δ: 8.72 (d, *J* = 2.4 Hz, 1H), 8.06 (dd, *J* = 8.4, 2.4 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 1H), 7.11 (bs, 1H), 2.45 (app q, *J* = 5.2 Hz, 2H), 2.63 (t, *J* = 6.0 Hz, 2H), 2.54 (q, *J* = 7.2 Hz, 4H), 1.00 (t, *J* = 7.2 Hz, 6H). MS: ES(+ve) *m/z* 258 (33%), 256 (100 %, M+H). Calculated mass for C₁₂H₁₉N₃OCl = 256.1217.

N-(2-(diethylamino)ethyl)-6-fluoronicotinamide (2)

General procedure A was applied to 6-fluoronicotinic acid (150 mg, 1.06 mmol) and *N,N*-diethylethylenediamine (0.17 mL, 1.16 mmol) followed by column chromatography with EtOAc:MeOH:NH₃ (10:1:0.1) to give the title compound as a clear oil (167.1 mg, 65 %). ¹H NMR (CDCl₃, 400 MHz) δ: 8.61 (d, *J* = 2.4 Hz, 1H), 8.25 (dt, *J* = 8.4, 2.8 Hz, 1H), 8.01 (bs, 1H), 7.00 (dd, *J* = 8.4, 2.8 Hz, 1H), 3.49 (app q, *J* = 5.2 Hz, 2H), 2.66 (t, *J* = 6.0 Hz, 2H), 2.57 (q, *J* = 7.2 Hz, 4H), 1.04 (t, *J* = 7.2 Hz, 6H). LSMS ES(+ve) *m/z* 241 (8 %), 240 (56 %, M+H⁺), 168 (9 %) 167 (100 %). Calculated mass for C₁₂H₁₉N₃OF = 240.1533.

N-(4-(butyl(methyl)amino)butyl)-6-chloronicotinamide (3)

General procedure A was applied to 6-chloronicotinic acid (1.0 g, 6.34 mmol) and *N*-butyl-*N*-methylbutane-1,4-diamine (1.1 g, 6.98 mmol) followed by column chromatography with EtOAc:MeOH:NH₃ (10:1:0.1) to give the title compound as a clear oil which crystallised on standing (1.44 g, 76 %). ¹H NMR (CDCl₃, 400 MHz) δ: 8.69 (d, *J* = 2.4 Hz, 1H), 8.42 (bs, 1H), 8.06 (dt, *J* = 8.4, 2.8 Hz, 1H), 7.37 (d, *J* = 8.4 Hz, 1H), 3.42 (q, *J* = 5.2 Hz, 2H), 2.36-2.28 (m, 4H), 2.15 (s, 3H), 1.71-1.67 (m, 2H), 1.68-1.60 (m, 2H), 1.36-1.31 (m, 2H), 1.26-1.20 (m, 2H), 0.85 (t, *J* = 7.25 Hz, 3H). LRMS ES(+ve) *m/z* 300 (32 %), 298.4 (100 %, M+H⁺). Calculated mass for C₁₅H₂₅N₃OCl = 298.1686.

***N*-(4-(butyl(methyl)amino)butyl)-6-fluoronicotinamide (4)**

General procedure A was applied to 6-fluoronicotinic acid (200 mg, 1.41 mmol) and *N*-butyl-*N*-methylbutane-1,4-diamine (246 mg, 1.58 mmol) followed by column chromatography with EtOAc:MeOH:NH₃ (10:1:0.1) to give the title compound as a clear oil which crystallised on standing (216 mg, 54 %). ¹H NMR (CDCl₃, 400 MHz) δ: 8.57 (d, *J* = 2.4 Hz, 1H, ArH-2), 8.40 (bs, 1H), 8.24 (dt, *J* = 7.2, 2.4 Hz, 1H), 6.98 (dd, *J* = 8.4, 2.8 Hz, 1H), 3.44 (q, *J* = 5.2 Hz, 2H), 2.41-2.32 (m, 4H), 2.19 (s, 3H), 1.73-1.70 (m, 2H), 1.69-1.63 (m, 2H), 1.40-1.30 (m, 2H), 1.28-1.22 (m, 2H), 0.82 (t, *J* = 7.2 Hz, 3H). LRMS ES(+ve) *m/z* 283 (18 %), 282 (100 %, M+H⁺). Calculated mass for C₁₅H₂₅N₃OF = 282.1982.

2-Chloro-*N*-[2-(diethylamino)ethyl]nicotinamide (5)

General procedure A was applied to 2-chloronicotinic acid (200 mg, 1.27 mmol) and *N,N*-diethylethylenediamine (0.2 mL, 1.39 mmol) followed by column chromatography with EtOAc:MeOH:NH₃ (5:1:0.1) to give the title compound as a

clear oil (226 mg, 69.5 %). ^1H NMR (CDCl_3 , 400 MHz) δ : 8.43 (dd, $J = 4.4, 2.0$ Hz, 1H), 8.10 (dd, $J = 7.6, 2.0$ Hz, 1H), 7.32 (dd, $J = 7.6, 4.8$ Hz, 1H), 7.26 (bs, 1H), 3.05 (q, $J = 4.8$ Hz, 2H), 2.64 (t, $J = 6.4$ Hz, 2H), 2.55 (q, $J = 7.2$ Hz, 4H), 1.00 (t, $J = 7.2$ Hz, 6H). LRMS ES(+ve) 258.3 (13 %, $\text{M}+\text{H}^+$), 256.2 (39 %), 185.1 (32 %), 183.0 (100 %). Calculated mass for $\text{C}_{12}\text{H}_{19}\text{N}_3\text{OCl} = 256.1217$.

2-Fluoro-N-[2-(diethylamino)ethyl]nicotinamide (6)

General procedure A was applied to 2-fluoronicotinic acid (200 mg, 1.41 mmol) and *N,N*-diethylethylenediamine (0.22 mL, 1.56 mmol) followed by column chromatography with EtOAc:MeOH: NH_3 (5:1:0.1) to give the title compound as a clear oil (232 mg, 68 %). ^1H NMR (CDCl_3 , 400 MHz) δ : 8.56 (ddd, $J = 10.0, 7.6, 2.0$ Hz, 1H), 8.29 (dt, $J = 4.8, 1.2$ Hz, 1H), 7.63 (bs, 1H), 7.33 (ddd, $J = 7.6, 4.8, 2.4$ Hz, 1H), 3.51 (q, $J = 5.2$ Hz, 2H), 2.64 (t, $J = 6.0$ Hz, 2H), 2.55 (q, $J = 7.2$ Hz, 4H), 1.03 (t, $J = 7.2$ Hz, 6H). LRMS ES(+ve) 241.2 (7.4 %), 240.0 (53 %), 168.0 (9.3 %), 167.1 (100 %). Calculated mass for $\text{C}_{12}\text{H}_{19}\text{N}_3\text{OF} = 240.1533$.

2-Chloro-N-[2-(diethylamino)ethyl]isonicotinamide (7)

General procedure A was applied to 2-chloroisonicotinic acid (200 mg, 1.26 mmol) and *N,N*-diethylethylenediamine (0.2 mL, 1.39 mmol), followed by column chromatography with EtOAc:MeOH: NH_3 (5-1-0.1) to give the title compound as a clear oil (210 mg, 65 %). ^1H NMR (CDCl_3 , 400 MHz) δ : 8.48 (d, $J = 5.2$ Hz, 1H), 7.64 (s, 1H), 7.49 (dd, $J = 5.2, 1.6$ Hz, 1H), 7.07 (bs, 1H), 3.40 (q, $J = 5.6$ Hz, 2H), 2.64 (t, $J = 5.6$ Hz, 2H), 2.58 (q, $J = 6.8$ Hz, 4H), 1.03 (t, $J = 6.8$ Hz, 6H). LRMS ES(+ve) 258.1 (21 %), 256 (64 %, $\text{M}+\text{H}^+$), 185 (32 %), 183 (100 %). Calculated mass for $\text{C}_{12}\text{H}_{19}\text{N}_3\text{OCl} = 256.1217$.

2-Fluoro-N-[2-(diethylamino)ethyl]isonicotinamide (8)

General procedure A was applied 2-fluoroisonicotinic acid (200 mg, 1.41 mmol) and *N,N*-diethylethylenediamine (0.22 mL, 1.56 mmol), followed by column chromatography with EtOAc:MeOH:NH₃ (5:1:0.1) to give the title compound as a clear oil (261 mg, 77%). ¹H NMR (CDCl₃, 400 MHz) δ: 8.33 (d, *J* = 5.2 Hz, 1H), 7.47 (dt, *J* = 5.2, 1.6 Hz, 1H), 7.27 (bs, 1H), 7.08 (bs, 1H), 3.48 (q, *J* = 5.2 Hz, 2H), 2.65 (t, *J* = 6.0 Hz, 2H), 2.57 (q, *J* = 7.2 Hz, 4H), 1.04 (t, *J* = 7.2 Hz, 6H). LRMS ES(+ve) 241.3 (14 %), 240.2 (100 %). Calculated mass for C₁₂H₁₉N₃OF = 240.1533.

II. Biological supporting information

a. Animal biodistribution methods

In vivo tumor model animal experiments were performed in compliance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Female C57BL/6J black and BALB/c nude mice (5 weeks) were obtained from the Animal Resources Centre (Western Australia). B16F0 murine melanoma cells were originally obtained from the European Collection of Cell Cultures (UK), and A375 human amelanotic melanoma cells were originally obtained from the American Type Culture Collection (USA). Before transplantation, B16F0 and A375 melanoma cells were maintained as a monolayer in RPMI culture medium supplemented with 10 % foetal calf serum (FCS) and antibiotics, and passaged with trypsinisation. Early passages were frozen and stored in liquid nitrogen. Cells were passaged to P=10 and then discarded. Frozen aliquots were grown in a monolayer culture to less than 100 % confluence and, for

transplantation, were trypsinised and washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS). For inoculation, B16F0 melanoma cells were resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS at 3×10^6 viable cells/mL, and 0.1 mL was subcutaneously injected into the left flank of 6- to 7-week-old C57BL/6J mice. Eleven days later, tumors could be palpated in 100 % of inoculated animals. A375 human melanoma cells were resuspended at 1×10^7 viable cells/mL, and 0.1 mL was injected subcutaneously into the left flank of 6-week-old BALB/c nude mice. Twenty-five to 26 days later, tumors could be palpated, with >90 % of inoculated animals developing tumors.

Biodistribution studies were performed 11 days (B16F0 melanoma) and 25 days (A375 melanoma) after tumor transplantation. The [^{18}F]nicotinamide derivatives (0.4 – 5.5 MBq, 100 μL) were injected intravenously into mice via the tail vein. Time points of 0.25, 0.5, 1, 3, 6 h after injection were chosen for determining the distribution of compounds in tumors and designated organs. Groups of mice ($n=5$) were sacrificed by CO_2 asphyxiation, weighed and dissected. Selected organs were collected, weighed and their radioactivity was measured with a γ -counter. The remaining activity in the carcass was also determined in order to obtain the total activity in the mouse at each time point. The fraction of injected activity [percent injected dose (% ID)] in the organ was calculated by comparison with suitable dilutions of the injected dose. Then, the radioactivity concentration in the organ (% ID/g) was found by dividing the percent injected dose for each organ by the weight of the organ. Contrast ratio values at time of sacrifice (CR_t) were calculated for each animal by dividing the radioactive tumor concentration by the mean concentration of radioactivity remaining in the mouse at the time of sacrifice t according to the following formula:

$CR_t = [\text{activity in tumor at time (t)} / \text{mass of tumor (g)}] / [\text{remaining activity in mouse at time (t)} / \text{weight of animal (g)}]$.

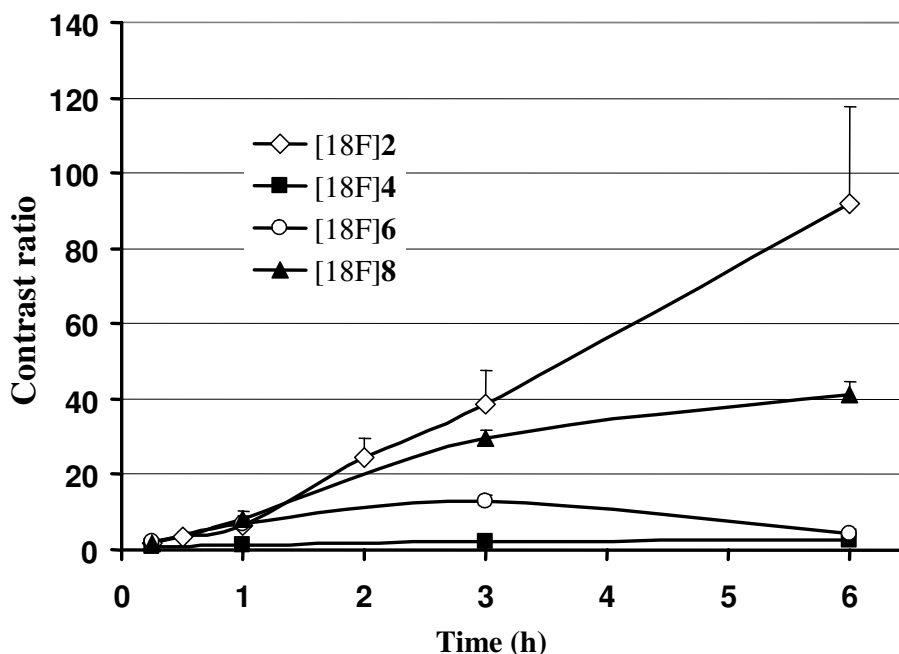


Figure 1 Contrast ratios ($CR \pm S.D.$; $n = 5$) after the injection of [^{18}F]fluoronicotinamide series in C57BL/6J black mice with B16F0 tumor.

b. General methods for imaging and autoradiography

B16F0 murine melanoma cells were maintained at $37^\circ\text{C} / 5\% \text{ CO}_2$ in RPMI /10% FCS. 1×10^6 or 2×10^6 cells in $50 \mu\text{L}$ of PBS were implanted subcutaneously on the flank of 8 week old C57BL/6J black mice. Ten days after implantation mice were injected via the lateral tail vein with 26 - 28 MBq of [^{18}F]2 in $100 \mu\text{L}$ saline. At 60 minutes post tracer injection, mice were anaesthetized by inhalation of $2.5\% \text{ isoflurane} / 50\% \text{ O}_2$ in air (flow rate $200 \text{ mL} / \text{min}$) and scanned in a prone position for 10 minutes on a Phillips Mosaic animal PET scanner (Phillips Medical Systems, USA). Following the scan, animals were sacrificed, the tumor removed and frozen in optimum cutting medium (OCT) medium. Sections ($40 \mu\text{m}$) were cut and scanned for

60 minutes in a Biospace MicroImager M40 high resolution autoradiography unit equipped with a digital camera (Biospace Lab, Paris, France).

c. PET imaging reconstruction

PET images were reconstructed using the 3-D RAMLA algorithm^{3,4} and images displayed using standard image software available on the Mosaic scanner. Tracer contrast was calculated as a tumor to background ratio (T:B) using region-of-interest (ROI) software on the Mosaic workstation. Briefly, ROIs were manually drawn around the tumor and an average background region on transaxial slice images displayed on the workstation. The background ROI was chosen to represent tracer present within the blood pool and non-tumor tissue, excluding regions of increased uptake such as spleen. Maximum and average counts per pixel within the ROI of each slice were automatically stored as data files. Uptake ratios were then calculated by dividing the maximum number of counts per pixel within a tumor ROI by the average number of counts per pixel within the background ROI.

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