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FULL PAPER

[^{18}F]PRIMATX, a new positron emission tomography tracer for imaging of autotaxin in lung tissue and tumour-bearing mice.

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Abstract: Autotaxin (ATX) is a secreted enzyme with tissue levels associated with tissue injury, which increase during wound healing and chronic fibrotic diseases. We selected [^{18}F]-(*R*,*E*)-3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl) phenyl)-1-(4-((5-(2-fluoroethoxy)pyridin-2-yl)methyl)-2-methylpiperazin-1-yl)prop-2-en-1-one ([^{18}F]PRIMATX, [^{18}F]**2**), a tracer for positron emission tomography, to image ATX expression *in vivo*. It successfully differentiates expression levels in lung tissue samples from idiopathic pulmonary fibrosis patients, and allows the detection of ATX-expressing tumours in living mice, confirming its potential for development as a clinical imaging agent.

Introduction

Autotaxin (ATX), also known as ectonucleotide pyrophosphatase/phosphodiesterase (ENPP2), is a secreted phosphodiesterase possessing lysophospholipase D activity.[1] It produces the bioactive lipid mediator lysophosphatidic acid (LPA), by hydrolysis of lysophosphatidylcholine (LPC) in serum.[2] Enpp2+/- heterozygotes have plasma LPA levels that are half that of wild-type mice.[2b] LPA is involved in the pathogenesis of a number of diseases, including cancer,[3] pulmonary fibrosis,[4] liver fibrosis,[5] and possibly neuropathic pain.[6]

LPA and ATX levels markedly increase following tissue injury, during wound healing and in chronic fibrotic diseases. In lung fibrosis elevated local LPA levels have been shown to play a role in the migration, differentiation and secretion profile of fibroblasts acting primarily via the G-protein coupled lysophosphatidic acid receptor LPA1. Samples of lung tissue from idiopathic pulmonary fibrosis (IPF) patients show increased levels of ATX expression.[4b] Likewise, ATX is over-expressed in multiple human cancer cell lines.[7] It was shown that ATX is associated with the initiation and progression of breast cancer in mice[8] and human,[9] and that ATX expression in human hepatocellular carcinoma is correlated with tumour grade as well as with the risk of death.[10] Autotaxin quantification by imaging in fibrosis or cancer patients, therefore, has the potential to become an important diagnostic and prognostic tool.

Non-invasive nuclear imaging techniques provide information about the physiology and biochemistry of experimental animals, patients and volunteers. They rely on the use of imaging instruments that can detect radiation emitted from a radioisotope bound to a physiologically relevant molecule (a radiotracer). Quantification of the emitted radiation allows the reconstruction of images revealing the distribution and concentration of the radiotracer as a function of time.[11] Positron emission tomography (PET) is a preferred technique as it offers a comparatively high spatial and temporal resolution. It involves the use of radiotracers labelled with positron emitting radionuclides such as carbon-11 (^{11}C) or fluorine-18 (^{18}F), both of which have short physical half-lives, allowing for same-day imaging. The system detects pairs of 511 keV gamma rays resulting from the annihilation of the emitted positron with a local electron.

A number of PET tracers have already been developed to image, quantify and monitor diseases. The most frequently used is 2-deoxy-2-[^{18}F]fluoroglucose ([^{18}F]FDG), which is useful to monitor glucose metabolism and identify hyper-metabolic cancerous lesions.[11] Further examples are tracers binding to the translocator protein (TSPO) on mitochondrial membranes for imaging inflammation,[12] and tracers for imaging Tau aggregates[13] or amyloid plaques,[14] the hallmarks of Alzheimer's disease.

Another important application of PET imaging studies is that, when conducted with an appropriate radiotracer, it facilitates the *in vivo*, non-invasive determination of the level of target engagement of a drug candidate without having to label the drug itself. This is particularly important when it comes to optimizing dose and regimen of a new molecular entity, and to precisely quantify the duration of its pharmacodynamic effects. While blood

sampling makes it easy to determine the duration of exposure in plasma, it only gives a very rough approximation of exposure in the organ of interest and ignores binding kinetics. In particular, multiple parameters such as the rate of tissue permeation, P-glycoprotein mediated efflux, and k_{off} influence target engagement in tumours. These parameters are complex to measure precisely, making predictions of clinical efficacy difficult and imprecise. Target occupancy studies allows addressing questions such as whether the drug has sufficiently penetrated the tissue of interest, and whether the selected dose achieves enough receptor occupancy for the desired effects, with an adequate safety margin. Selecting this approach to dose selection in drug development will help reduce the risk of drug failure due to lack of sufficient target engagement.

The lead structure for PET tracer optimization (**1**, Figure 1) originates in the modification of a hit from a high-throughput screen.[15] Optimized as a drug candidate, **1** displays a high affinity for ATX, and PET tracer-like properties. It however displays an elimination half-life that is too long for a potential PET tracer ($t_{½}$ = 1.1h in male Sprague-Dawley rats after 1 mg/kg i.v,), especially if labelled with ^{11}C ($t_{½}$ = 20.3 min). To address this issue, we sought to identify a fluorine-containing derivative with a shorter half-life, for labelling with ^{18}F ($t_{½}$ = 109.8 min). We also explored structural modifications to attempt increasing affinity further. Several suitable candidates were identified. Compound **2** (PRIMATX) was selected for characterization of its imaging potential.

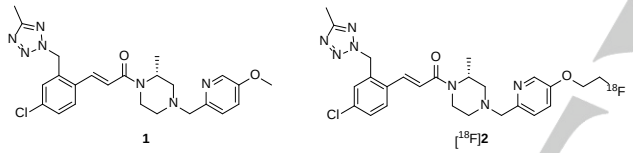
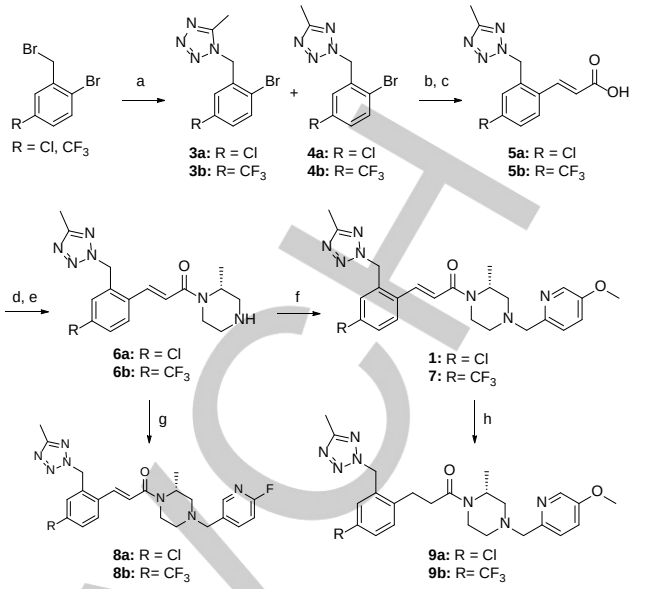


Figure 1. Structure of lead **1** and radiolabelled derivative [^{18}F]**2**.

Results and Discussion

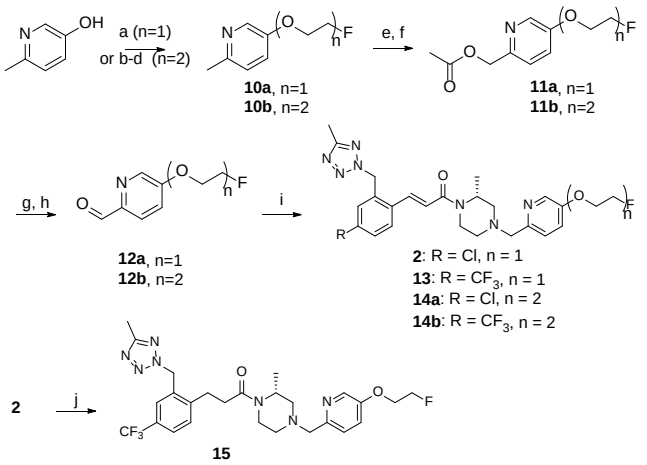
Synthesis

Scheme 1 shows the synthetic route toward compounds **1**, its CF_3 analogue (**7**), as well as variations of the pyridine ring (**8a,b**) and saturation of the double bond (**9a,b**). Alkylation of 1-bromo-2-(bromomethyl)-4-chlorobenzene or 1-bromo-2-(bromomethyl)-4-(trifluoromethyl)-benzene with 5-methyl-2H-tetrazole led to a mixture of regioisomers. Subsequent isolation by filtration gave the desired compounds **4a,b**, which were reacted with ethyl acrylate and hydrolyzed to provide acids **5a,b**. Carboxylic activation by HATU and coupling with a partially protected piperazine, followed by deprotection, furnished intermediates **6a,b**. Condensation with picolinaldehyde in the presence of acetic acid and 2-picoline-borane as reducing agent gave target compounds **1** and **7**. The 6-fluoropyridinyl analogues **8a,b** were prepared by condensation of **6a,b** with 6-fluoronicotinaldehyde, while a Pd/C catalyzed hydrogenation of **1** and **7** yielded **9a** and **9b**, respectively.



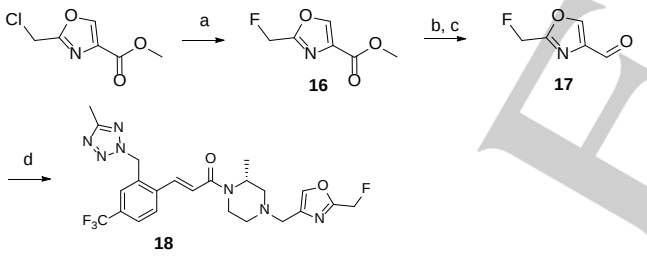
Scheme 1. Experimental condfitions: a) K_2CO_3, DMF, RT, 2 h, **4a**: 37%, **4b**: 37%; b) P(o-tol)$_3$, NEt_3, DMF, $Pd(OAc)_2$, 100°C, 18 h; c) 2M NaOH, EtOH, RT, 18 h, quant.; d) HATU, DIPEA, NMP, RT, 2h; e) TFA, CH_2Cl_2, RT, 4 h, 6a: 95%, 6b: 84%; f) 5-methoxypicolinaldehyde, 2-picoline-borane, AcOH, MeOH, RT, 16h, **7**: 55%; g) 2-fluoro-5-formylpyridine, 2-picoline-borane, AcOH, MeOH, RT, 16h, **8a**: 13%, **8b**: 16%; h) **9a**: Pt/C, EtOH, H_2, RT, 21 h, 21%; **9b**: Pd/C, EtOH, H_2, RT, 17 h, 84%.

Scheme 2 shows the preparation of picolinaldehydes **12a,b**, and their coupling to **6a,b** to provide the two pairs of analogues **2**, **13** and **14a,b**. Treatment of 6-methylpyridin-3-ol with 2-fluoroethyl 4-methylbenzene sulfonate[16] in the presence of potassium carbonate and sodium iodide led to **10a**. The methyl group of **10a** was oxidized following a published synthetic sequence[17] to yield aldehyde **12a**, which was condensed with **6a,b** in the presence of acetic acid and 2-picoline-borane to give target compounds **2** and **13**. Alternatively, alkylation of 6-methylpyridin-3-ol with 2-(2-chloroethoxy)ethan-1-ol and subsequent treatment with tetrabutylammonium fluoride gave **10b**. Oxidation of the methyl group led to **12b**, which was condensed with **6a** or **6b** to provide target compounds **14a,b**. Finally, Pd/C catalyzed hydrogenation of **13** yielded the saturated analogue **15**.



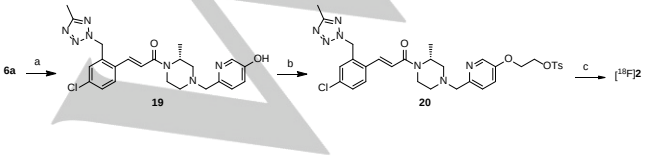
Scheme 2. Experimental conditions: a) FEtOTs, K_2CO_3, NaI, DMF,80°C, 16 h, **10a**: 83%, **10b**: 85%; b) 2-(2-chloroethoxy)ethan-1-ol, K_2CO_3, NaI, DMF, 85°C, 16 h; c) p-tolylsulfonyl chloride, CH_2Cl_2, RT, 16 h; d) TBAF, THF, 65°C, 30 min; e) m-CPBA, $CHCl_3$, 0°C, 3 h; f) Ac_2O, 130°C, 30 min; g) NaOH, H_2O, EtOH, reflux, 1 h; h) MnO_2, CH_2Cl_2, RT, 2 days; i) AcOH, MeOH, 2-picoline borane, RT, 16 h, **2**: 23%, **13**: 58%, **14a**: 50%, **14b**: 49%; j) Pd/C, H_2, EtOH, 18 h, 47%.

The oxazole-carbaldehyde **17** was prepared according to scheme 3. Fluorination of methyl 2-(chloromethyl)oxazole-4-carboxylate led to compound **16**. Consecutive reduction and oxidation led to aldehyde **17**, whose condensation with **6b** afforded compound **18**.



Scheme 3. Experimental conditions: a) TBAF, CH_3CN, 21 hours, RT, 32%; b) DIBAL-H, THF, -78°C, time, 41%; c) MnO_2, CH_2Cl_2, RT, 18 hours; d) **6b**, AcOH, MeOH, 2-picoline borane, RT, 16 h, 24%.

The precursor for the radiosynthesis of [^{18}F]**2** was prepared according to Scheme 4. Condensation of **6a** with 5-hydroxypicolinaldehyde led to compound **19**. Further treatment with ethane-1,2-diyl bis(4-methylbenzenesulfonate) and cesium carbonate provided **20**, the precursor for the radiosynthesis of [^{18}F]**2**. The latter was obtained after treatment with [^{18}F]KF in presence of Kryptofix-222.



Scheme 4. Radiosynthesis of [^{18}F]**2**: a) 5-hydroxypyridine-2-carboxaldehyde, $NaBH(OAc)_3$, CH_3COOH, $(ClCH_2)_2$, RT, 17h; b) ethylene di(*p*-toluenesulfonate), Cs_2CO_3, DMF, RT, 2 h, 62%; c) [^{18}F]KF, crypt-222, DMSO, 140°C, 10 min.

Optimization and pharmacological characterization

Starting from structure **1**, we initially explored the exchange of the aromatic chlorine by a trifluoromethyl group. This modification was tolerated, only leading to a slight decrease in affinity (**7**). Replacing the 5-methoxypyridin-2-yl group by a 6-fluoropyridin-3-yl (**8a**), which would allow an easy radiolabelling strategy, was less favorable. Interestingly, the combination of these two modifications led to a partial recovery of affinity (**8b**). Reduction of the double bond (**9a**, **9b**) did not bring any advantage, with both derivatives being less potent that the lead structure.

To introduce another option for radiolabelling, we extended the methoxy group to a fluorine-containing side-chain, envisaging the introduction of ^{18}F from the corresponding tosylate precursor. We first prepared the fluoroethyl analogues **2** and **13**, which proved to have affinities equivalent to the lead structure. Extending the side-chain to a fluoroethoxyethoxy group had a minimal effect on affinity (**14a,b**). Further variations, as exemplified by **15** and **18**, identified additional derivatives with high affinity and similar overall properties. The good permeability and low efflux ratio of these molecules in the MDCK-MDR1 assay hints at a good organ distribution, including a high likelihood of brain penetration. Their properties appeared promising and only differed minimally, so we selected the lowest molecular-weight compound (**2,** MW = 514) for further evaluation. Furthermore, the radiosynthesis of the radiolabelled [^{18}F]**2** was expected to be straightforward.

Table 1. Compound list and *in vitro* properties.

Cmpd	IC_{50} (nM)	CHI(IAM)[a]	logD[b]	%HSA[c]	flux[d]	efflux ratio[]
1	3	-	-	-	12.5	1.0
2	4	35	3.0	91	8.1	1.9
7	10	35	3.2	91	14.1	1.0
8a	51	36	3.3	93	7.4	1.1
8b	13	36	3.5	94	8.3	1.1
9a	22	34	2.9	88	12.9	1.2
9b	15	35	3.2	89	26.3	0.9
13	3	35	3.2	91	7.7	1.6
14a	6	34	3.2	88	10.4	3.4
14b	5	33	3.0	87	-	-
15	3	-	3.1	87	10.3	1.3
18	2	33	2.8	86	13.8	1.8

[a] Chromatographic Hydrophilicity Index (CHI) measured by HPLC on Immobilized Artificial Membranes (IAM)[18]; [b] logD measured in octanol/buffer pH 7.4; [c] ; [d] measured in the Madin Darby Canine Kidney (MDCK) cell line transfected with the human multidrug resistance (MDR1) gene.

The selectivity of **2** was tested at a concentration of 30 μM in a panel of 63 pharmacological targets from the central nervous system and periphery; no activity was found (results not shown). Its CHI(IAM) value of 35, similar to all compounds in this series, indicates a very low propensity to bind to cell membranes, predicting minimal non-specific binding *in vivo*.[19] The rat plasma protein free fraction was determined to be 5%. Finally, the pharmacokinetic properties of the tracer candidate (Table 2) indicate a faster elimination than the lead compound, with a plasma half-life of 0.7 h. It also shows a good exposure in the lung, an organ of interest for pulmonary fibrosis. A separate

pharmacokinetics study showed that in rats, brain penetration was modest, with a brain/blood ratio of 0.24, five minutes after intravenous administration (1 mg/kg). In combination with a high affinity for ATX and a low non-specific binding, this is nevertheless expected to be sufficient for PET imaging of the central nervous system.

Table 2. Pharmacokinetic properties of **2**, one hour after intravenous administration (1 mg/kg) in Sprague Dawley male rats (n=3). Formulation as a solution in 1% 1M HCl, 10% PEG300, 25% (20% solutol), 64% PBS.

Blood (nM)[a]	Lung (nM)[a]	Lung/blood ratio[a]	t½ (h)	CL[a] (ml/min/kg)	Vss[b] (L/kg)
55±13	241±80	4.6	0.7±0.1	23±11	1.0±0.3

[a] CL = clearance; [b] Vss = Distribution volume at steady state

Encouraged by this profile, we moved to the characterization of the radiolabelled tracer, [^{18}F]**2.** We first performed immunohistochemistry for ATX in human IPF lung samples, to qualitatively estimate the level of expression of ATX in diseased tissues (Figure 2). One sample showed a low level of ATX expression, while the other two showed a robust signal, visible under the microscope as a diffuse brown staining, as expected for a secreted protein.

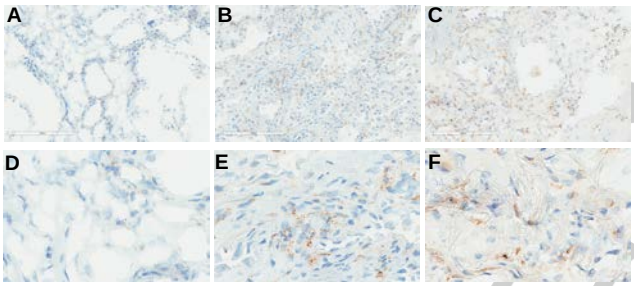


Figure 2. Immunohistochemistry with anti-ATX antibody on human fibrotic lung sections. Upper panel: A, lung with low expression of ATX; B and C, lungs with high expression of ATX. Lower panel, higher magnifications of image from upper panel showing brown staining of secreted ATX.

A subsequent *in vitro* immersion autoradiography on adjacent lung sections, both under control and self-blocking conditions (Figure 3), confirmed the ability of [^{18}F]**2** to detect the increase in autotaxin expression associated with fibrosis.[4] The first section (A) was shown to have a moderate autotaxin expression by immuno-histochemistry (IHC), while sections B and C have a high level of expression. Self-blocking decreased signal intensity significantly, providing a first indication of high selectivity of uptake and that [^{18}F]**2** might be suitable for the intended purpose.

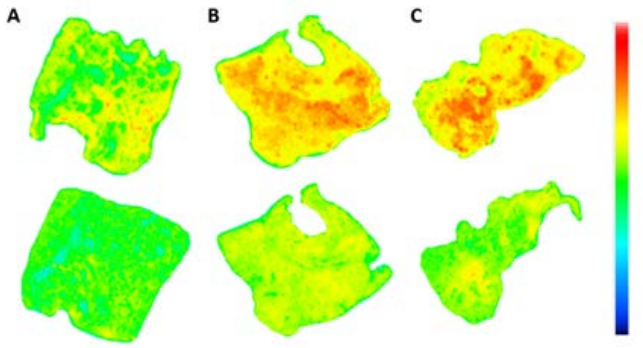


Figure 3. *In vitro* autoradiography with [^{18}F]**2** on human fibrotic lung sections, logarithmically scaled. Upper sections: total binding; lower sections: binding after blocking with 10 μM **2**; a) section with moderate ATX expression; b) and d) sections with high ATX expression as determined via IHC.

After failing to reliably and reproducibly induce lung fibrosis using the bleomycin model in mice,[20] and in view of the scarcity of alternative fibrosis animal models, we decided to pursued the *in vivo* evaluation of [^{18}F]**2** in mice bearing autotaxin over-expressing tumours. Our aim was to confirm that the tracer is able to image its target *in vivo*, and that its signal can be blocked by pre-treatment with a structurally different autotaxin inhibitor.

Four B6D2F1 mice inoculated with subcutaneous B16F10 melanoma tumours (140 – 391 mm^3) were injected with [^{18}F]**2** (1.09 ± 0.33 MBq, < 200 μL). The animals underwent a 60-minute whole body dynamic PET scan, followed by computer tomography (CT) for anatomical reference. The following day, the same four animals were administered 100 mg/kg **21**[11] as blocking agent, via oral gavage. Thirty minutes later, animals were injected with [^{18}F]**2** (10.5 ± 0.22 MBq, < 200 μL) and underwent a second 60-minute whole body dynamic PET scan, followed by a CT scan for anatomical reference.

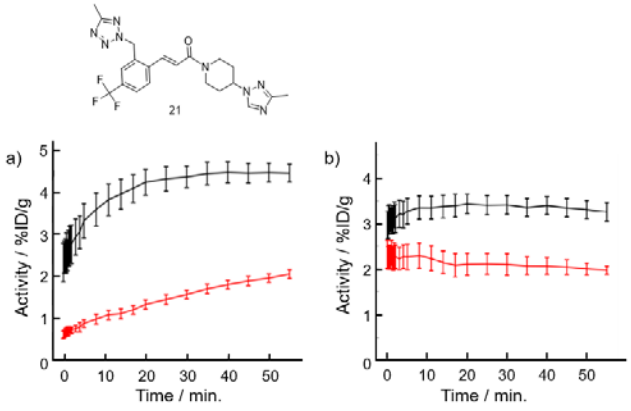


Figure 4. Structure of blocker **21** and time course of percent injected dose per gram (%ID/g, mean +/- SEM) in a) tumour; b) brain. Black lines represent baseline signals, red lines represent signal after blocking with 100 mg/kg **21**.

[^{18}F]**2** shows an average uptake of 4.4 %ID/g in B16F10 tumours (Figure 4). The uptake was decreased to 2.1 %ID/g after blocking, indicating that approximately 60% of uptake is antigen specific. In the brain, average uptake was 3.4 %ID/g, and decreased by

39% to 2.1 %ID/g upon blocking, confirming sufficient brain penetration for imaging purposes. The distribution of radioactivity suggests that [^{18}F]**2** and its metabolites are mostly excreted by a combination of renal and hepatic clearance (Figure 5).

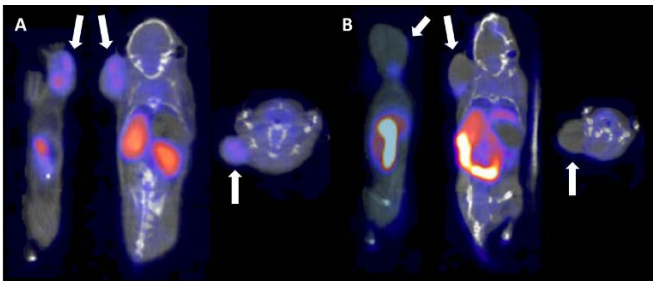


Figure 5 Representative averaged PET image, scaled to 0-35 %ID/g (measured between 20 and 55 minutes after [^{18}F]**2** injection), tumour centred, 60 min.; a) baseline; b) blocked; white arrows point to tumour.

Conclusions

The optimization of lead compound **1** led to the selection of its fluorinated derivative **2**, a very selective, high-affinity and permeable autotaxin ligand, as a promising PET tracer candidate with low non-specific binding. Pharmacokinetic studies demonstrated good penetration into lung and a shortened half-life of 0.7h, compatible with the requirements for a PET imaging agent radiolabelled with fluorine-18. Immersion autoradiographic studies in human fibrotic lung tissue confirm the ability of [^{18}F]**2** to differentiate areas of low and high autotaxin expression. A subsequent *in vivo* PET study in mice bearing autotaxin-expressing B16F10 tumours demonstrated the potential of this tracer for tumour imaging. A clear imaging signal associated with the tumour was blocked by a structurally different autotaxin ligand. It is, to our knowledge, the first time an autotaxin tracer successfully demonstrate the possibility to image melanoma cancer cells *in vivo*, confirming the relevance of this target for diagnostic purposes. Taken together, these results confirm that [^{18}F]**2** fulfils the requirement of a PET tracer candidate for further clinical development.

Experimental Section

General methods: All chemicals, reagents, and solvents for the synthesis of the compounds were analytical grade, purchased from commercial sources and used without purification, unless otherwise specified. ^{1}H NMR spectra were acquired on a Bruker 400 MHz instrument. Chemical shift (δ) values are reported in parts per million (ppm) relative to the residual solvent peak.

Liquid chromatography/mass spectrometry (LC/MS) analyses were run as follows (% = percent per volume): Method A (t_{RA} = retention time A): UPLC-ZQ2000, column: Acquity HSS-T3 1.8 μm, 2.1 x 50 mm, 60°C; mobile phase: H_2O + 0.05% H_2CO_2H + 3.75 mM $CH_3CO_2NH_4$ (A) / CH_3CN + 0.04% HCO_2H (B); gradient: from 5 to 98% B in 1.4 min, then 0.7 min isocratic; flow rate: 1.0 mL.min^{-1}. Method B (t_{RB} = retention time B): UPLC-ZQ2000, column: Acquity HSS-T3 1.8 μm, 2.1 x 50 mm, 60°C; mobile phase: H_2O + 0.05% H_2CO_2H + 3.75 mM $CH_3CO_2NH_4$ (A) / CH_3CN + 0.04% HCO_2H (B); gradient: from 5 to 98% B in 9.4 min, then 0.7 min isocratic; flow rate: 1.0 mL.min^{-1}. Preparative high-pressure liquid chromatography (HPLC) purifications were run as follows (% = percent per volume): Method C: Gilson Trilution LC, column: SunFire C18OBD 5 μm, 30 x 100 mm; mobile phase: H_2O + 0.1% TFA) (A) / CH_3CN + 0.1% TFA (B); gradient: from 5 to 50% B in 20 min; flow rate: 40 mL min^{-1}.

2-(2-Bromo-5-chlorobenzyl)-5-methyl-2H-tetrazole (4a): 5-Methyl-2H-tetrazole (77 g, 913 mmol) was placed in a flask with dry DMF (400 mL) at 0°C using an ice bath. Potassium carbonate (168 g, 1217 mmol) was added in portions followed by dropwise addition of 1-bromo-2-(bromomethyl)-4-chlorobenzene (173 g, 608 mmol) in DMF (400 mL). The resulting mixture was stirred at room temperature for 2 h. The mixture was poured into water and the resulting suspension collected by filtration. The solid was triturated with iso-hexane and the undissolved solid was removed by filtration. The filtrate was concentrated under reduced pressure giving a white solid which was suspended in water and stirred overnight. The product was filtered and washed with water to afford the desired product (64.9 g, 37 %), which was used in the next step without further purification. LC/MS: t_{RA}=1.15 min, m/z: 289.0 [M+H].

2-(2-Bromo-5-(trifluoromethyl)benzyl)-5-methyl-2H-tetrazole (4b): To a stirred solution of 5-methyl-2H-tetrazole (19.44 g, 231 mmol) in DMF (154 mL) at 10°C under N_2 was added K_2CO_3 (42.6 g, 308 mmol). The resulting suspension was cooled to - 2°C (ice salt bath) and a solution of 1-bromo-2-(bromomethyl)-4-(trifluoromethyl)benzene (49 g, 154 mmol) in DMF (66 mL) was added dropwise over 30 min keeping the internal temperature below 5 °C. The mixture was then allowed to warm to room temperature and the resulting white suspension stirred overnight. Water (400 mL) was added slowly to the mixture which was then extracted with EtOAc (2 x 500 mL). The combined organic extracts were washed with brine (500 mL), dried ($MgSO_4$) and concentrated in vacuo to yield a colourless oil. Iso-hexane (150 mL) was added and the resulting slurry was filtered and the solid washed with iso-hexane (2 x 50 mL). The filtrate was concentrated in vacuo to yield a colourless oil. Purification by chromatography on silica gel eluting with 0 to 50% EtOAc in iso-hexane afforded the title compound (18.4 g, 37%). LC/MS: t_{RA} =1.30 min, m/z: 321.3 [M+H]. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 2.58 (3 H, s), 5.83 (2 H, s), 7.11 (d, *J* = 2.46 Hz, 1H), 7.23 (dd, *J* = 8.5 Hz, 11.9 Hz, 1H), 7.57 ppm (d, *J* = 8 Hz, 1H).

(*E*)-Ethyl 3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl) acrylate: Compound **4a** (15 g, 52.2 mmol), tri-o-tolylphosphine (0.79 g, 2.61 mmol) and triethylamine (10.56 g, 104 mmol) were placed in a flask with dry, degassed DMF (80 mL). Ethyl acrylate (7.83 g, 78 mmol) was added, followed by palladium diacetate (0.59 g, 2.61 mmol) and the reaction mixture was stirred at 100°C overnight. The mixture was allowed to cool to room temperature, diluted with EtOAc (150 mL) and filtered to remove solids, then partitioned between EtOAc and water. The organic phase was washed with water and brine, dried over $MgSO_4$, filtered, and the solvent was removed *in vacuo*. When ~75% of the solvent was removed, a solid precipitated out which was collected by filtration and dried to afford the title compound as a white solid (8.5 g, 53 %). LC/MS: t_{RA} =1.33 min, m/z: 307.5 [M+H]. ^{1}H NMR (400 MHz, DMSO-d6): δ 7.92 (1 H, d), 7.89 (1 H, d), 7.59 (1 H, d), 7.51 (1 H, d of d), 6.59 (1 H, d), 6.09 (2 H, s), 4.20 (2 H, q), 2.41 (3 H, s), 1.26 ppm (3 H, t).

(*E*)-3-(4-Chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)acrylic acid (5a): (*E*)-Ethyl 3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl) phenyl)acrylate (8.75 g, 28.5 mmol) was placed in a flask with EtOH (100 mL). 2M aq. NaOH (57.1 mL, 11 mmol) was added and the reaction mixture stirred at room temperature overnight. The ethanol was removed *in vacuo* and the reaction mixture acidified with 2M aq. HCl. The resulting precipitate was collected by filtration, washed with water and dried to afford the title compound as a white solid. It was used in the next step without further purification. LC/MS: t_{RA} =0.99 min, m/z: 279.2 [M+H].

(*E*)-Ethyl 3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4- (trifluoromethyl) phenyl)acrylate: To a stirred solution of **4b** (17 g, 52.9 mmol) in DMF (76 mL) was added tri-o-tolylphosphine (0.81 g, 2.65 mmol) and triethylamine

(14.76 mL, 106 mmol). The solution was de-gassed by bubbling N_2 through it for 20 min. $Pd(OAc)_2$ (0.59 g, 2.65 mmol) and ethyl acrylate (8.66 mL, 79 mmol) were added and the reaction mixture heated to 90 °C under N_2. After cooling to room temperature, the mixture was partitioned between water (150 mL) and EtOAc (250 mL). The phases were separated and the aqueous phase extracted with more EtOAc (250 mL). The combined organic layers were washed with brine (2 x 250 mL), dried over anhyd. $MgSO_4$ and concentrated in vacuo to yield the title compound as orange oil (5.2 g, 28.7%), and used as such in the next step.LC/MS: t_{RA}=1.36 min, m/z: 341.5 [M+H].

(*E*)-3-(2-((5-Methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl) phenyl)acrylic acid (5b): To a stirred solution of crude (E)-ethyl 3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)acrylate (18.02 g, approx. 53 mmol) in EtOH (212 mL) was slowly added 2M aq. NaOH (79 mL, 159 mmol). The resulting orange solution was stirred at room temperature overnight, then concentrated in vacuo to a volume of 100 ml, and then filtered. 5M HCl (38 mL) was added slowly to adjust the pH to 2 whereupon a solid started to crystallize out of solution. The mixture was stirred at room temperature for 2 h to allow full crystallization. The resulting slurry was filtered, and the filter cake washed with 50% aq. EtOH (2 x 20 mL). The solid was dried in vacuo at 40 °C overnight to afford the title compound, which was directly used in the next step. LC/MS: t_{RA}=1.14 min, m/z: 313.4 [M+H].

(*R*,*E*)-*tert*-Butyl 4-(3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)acryloyl)-3-methylpiperazine-1-carboxylate: HATU (2.11 g, 5.55 mmol) was added to a solution of compound **5a** in NMP (15 mL) and the mixture stirred at room temperature for 5 minutes. (*R*)-*tert*-butyl-3-methylpiperazine-1-carboxylate (0.93 g, 4.63 mmol) was added followed by DIPEA (1.62 mL, 9.26 mmol). The reaction mixture was stirred at room temperature for 2 h, then was poured into water and extracted with EtOAc. The organic portion was washed with water, sat. aq. sodium bicarbonate, water, brine, and dried over a phase separator. The solvent was removed under reduced pressure. Purification of the crude product by chromatography on silica using a gradient from 0 to 100% EtOAc in iso-hexane afforded the title compound (2.0 g, 95 %). LC/MS: t_{RA} =1.23 min, m/z: 461.3 [M+H]. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 1.20 (d, *J* = 7.09 Hz, 3 H), 1.42 (s, 9 H), 1.61 (s, 1 H), 1.90 - 2.00 (m, 2 H), 2.23 - 2.34 (m, 2 H), 2.45 (s, 3 H), 2.65 (s, 1 H), 2.77 (s, 3 H), 2.94 - 3.10 (m, 1 H), 3.31 (t, *J* = 7.03 Hz, 2 H), 5.72 (s, 2 H), 6.73 (d, *J* = 15.28 Hz, 1 H), 7.18 (d, *J* = 1.96 Hz, 1 H), 7.28 (dd, *J* = 8.44, 2.08 Hz, 1 H), 7.43 (d, *J* = 8.44 Hz, 1 H), 7.91 ppm (d, *J* = 15.28 Hz, 1 H).

(*R*,*E*)-3-(4-Chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)-1-(2-methylpiperazin-1-yl)prop-2-en-1-one (6a): To (*R*,*E*)-*tert*-butyl 4-(3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)acryloyl)-3-methylpiperazine-1-carboxylate (2.1 g, 4.56 mmol) in CH_2Cl_2 (22 mL) was added TFA (4.21 mL, 54.7 mmol) and the mixture was stirred at room temperature for 4 h. The solvent was removed under reduced pressure. The resulting residue was loaded onto an Isolute® SCX-2 cartridge eluting with MeOH followed by 2 M NH_3 in MeOH. The fractions were concentrated under reduced pressure to afford the title compound (1.2 g, 95%). LC/MS: t_{RA} = 0.64 min, m/z = 361.6 [M+H]. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 1.40 (d, J = 7.09 Hz, 3 H), 2.45 (s, 3 H), 2.76 - 2.95 (m, 1 H), 3.09 (d, *J* = 2.69 Hz, 2 H), 3.17 - 3.53 (m, 2 H), 4.05 - 4.82 (m, 2 H), 5.72 (s, 2 H), 6.70 (d, *J* = 15.28 Hz, 1 H), 7.25 (d, *J* = 2.08 Hz, 1 H), 7.30 (dd, *J* = 8.38, 2.02 Hz, 1 H), 7.43 (d, *J* = 8.31 Hz, 1 H), 7.93 ppm (d, *J* = 15.28 Hz, 1 H).

(*R*,*E*)-*tert*-Butyl 3-methyl-4-(3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)acryloyl)piperazine-1-carboxylate: T3P® 50% in ethyl acetate (4.5 mL, 7.7 mmol) was added to a solution of **5b** (2 g, 6.41 mmol), (*R*)-*tert*-butyl 3-methylpiperazine-1-carboxylate (2.0 g, 6.4 mmol) and NEt_3 (3.6 mL, 25.6 mmol) in CH_2Cl_2 (20 mL) and the resulting mixture stirred for 1 h at room temperature. The reaction mixture was diluted with sat. aq. sodium bicarbonate (100 mL) The aqueous solution was extracted with ethyl acetate (3x100 mL). The combined organic solutions were washed with water (50 mL), brine (50 mL), dried over sodium sulphate, filtered and concentrated in vacuo. Purification was performed by silica gel column chromatography eluting with a gradient of iso-hexane to ethyl acetate. The product fractions were combined and evaporated in vacuo to give a white solid. LC/MS: t_{RA}=1.39 min, m/z: 395.3 [M-100+H] which was directly used in the next step.

(*R*,*E*)-3-(2-((5-Methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)-1-(2-methylpiperazin-1-yl)prop-2-en-1-one (6b): TFA (10 mL) was added to a solution of (*R*,*E*)-*tert*-butyl 3-methyl-4-(3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl) acryloyl)piperazine-1-carboxylate (2.7 g, 5.46 mmol) in CH_2Cl_2 (10 mL) and the resulting mixture stirred for 1 h. Toluene (100 mL) was added and the reaction concentrated in vacuo. The resulting gum was stirred in diethyl ether (250 mL), water (1mL) was added and the resulting solid was collected by filtration, washed with ether and dried under vacuum to give the title compound as a trifluoroacetate salt (1.8 g, 84%). LC/MS: t_{RA}=0.74 min, m/z: 395.0 and 397.5 [M+H]. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 1.40 (d, *J* = 7.09 Hz, 3 H), 2.45 (s, 3 H), 2.76 - 2.95 (m, 1 H), 3.09 (d, *J* = 2.69 Hz, 2 H), 3.17 - 3.53 (m, 2 H), 4.05 - 4.82 (m, 2 H), 5.72 (s, 2 H), 6.70 (d, *J* = 15.28 Hz, 1 H), 7.25 (d, *J* = 2.08 Hz, 1 H), 7.30 (dd, *J* = 8.38, 2.02 Hz, 1 H), 7.43 (d, *J* = 8.31 Hz, 1 H), 7.93 ppm (d, *J* = 15.28 Hz, 1 H).

(*R*,*E*)-1-(4-((5-methoxypyridin-2-yl)methyl)-2-methylpiperazin-1-yl)-3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl) prop- 2-en-1-one (7): To a solution of compound **6b** (62 mg, 0.16 mmol) in MeOH (1.4 mL) were added AcOH (0.14 mL) and 5-methoxy-2-pyridinecarboxaldehyde (32.3 mg, 0.24 mmol). After stirring for 5 min, 2-picoline borane (26.6 mg, 0.25 mmol) was added. The reaction mixture was stirred at RT for 16 h. The volatiles were evaporated and the residue purified by flash chromatography (mobile phase: CH_2Cl_2 (A) / MeOH (B); gradient: 100% A to 10% B in 15 min). The desired fractions were concentrated to give the product as an orange oil (46 mg, 55%). LC/MS: t_{RA}= 0.9 min, m/z: 517.2 [M+H]. ^{1}H NMR (400 MHz, DMSO) δ= 1.16 - 1.30 (m, 4 H), 1.93 - 2.22 (m, 2 H), 2.41 (s, 3 H), 2.64 - 2.73 (m, 1 H), 2.85 (d, *J* = 10.51 Hz, 1 H), 3.45 - 3.67 (m, 2H), 3.83 (s, 3 H), 3.93 - 4.29 (m, 1 H), 4.34 - 4.76 (m, 1 H), 6.12 (d, *J* = 2.08 Hz, 2 H), 7.22 (d, *J* = 15.28 Hz, 1 H), 7.32 - 7.52 (m, 2 H), 7.67 - 7.92 (m, 3 H), 8.06 (d, *J* = 8.07 Hz, 1 H), 8.14 - 8.30 ppm (m, 1 H).

(*R*,*E*)-3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)-1-(4-((6-fluoropyridin-3-yl)methyl)-2-methylpiperazin-1-yl)prop-2-en-1-one (8a): To a solution of compound **6a** (120 mg, 0.33 mmol) in MeOH (3.0 mL) were added AcOH (0.3 mL) and 2-fluoro-5-formylpyridine (65.0 mg, 0.49 mmol). After stirring for 5 min, 2-picoline borane (56.4 mg, 0.53 mmol) was added. The reaction mixture was stirred at RT for 16 h and was concentrated. The crude product was purified by reversed-phase preparative HPLC (method C) to give 25 mg of a white powder (13%). LC/MS: t_{RA}=1.01 min, m/z: 470 [M+H]. ^{1}H NMR (400 MHz) δ= 1.28 (dd, *J* = 6.78, 2.26 Hz, 4 H), 2.07 - 2.36 (m, 2 H), 2.41 - 2.47 (m, 3 H), 2.64 - 2.81 (m, 1 H), 2.88 (br. s., 1 H), 3.65 (br. s., 2 H), 4.15 (d, *J* = 13.18 Hz, 1 H), 4.55 (br. s., 1 H), 5.96 (s, 2 H), 7.03 (d, *J* = 15.31 Hz, 1 H,) 7.14 (d, *J* = 8.41 Hz, 1 H), 7.45 - 7.54 (m, 2 H), 7.71 (d, *J* = 15.31 Hz, 1 H), 7.83 (s, 1 H), 7.95 (br. s., 1 H), 8.20 ppm (br. s., 1 H).

(*R*,*E*)-1-(4-((6-fluoropyridin-3-yl)methyl)-2-methylpiperazin-1-yl)-3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)prop-2-en-1-one (8b): AcOH (0.28 mL) and 2-fluoro-5-formylpyridine (59.5 mg, 0.46 mmol) were added to a solution of compound **6b** (120 mg, 0.30 mmol) in MeOH (2.8 mL). After stirring for 5 min, 2-picoline borane (51.6 mg, 0.49 mmol) was added. The reaction mixture was stirred at RT for 16 h and concentrated. The crude product was purified by flash chromatography: mobile phase: cyclohexane (A) / EtOAc (B); gradient: A to B in 20min, to provide 26 mg as a white solid (16%). LC/MS: t_{RA}=1.06 min, m/z: 504 [M+H]. ^{1}H NMR (400 MHz, DMSO) δ= 1.23 (d, *J* = 5.50 Hz, 3 H), 1.90 - 2.24 (m, 2 H), 2.42 (s, 3 H), 2.62 - 2.74 (m, 1 H), 2.84 (d, *J* = 10.27 Hz, 1 H), 3.44 - 3.69 (m, 2 H), 3.95 - 4.32 (m, 1 H), 4.37 - 4.71 (m, 1 H), 6.12 (d, *J* = 1.96 Hz, 2 H), 7.12 - 7.28 (m, 2 H), 7.72 - 7.89 (m, 3 H),

7.96 (td, *J* = 8.22, 2.38 Hz, 1 H), 8.07 (d, *J* = 8.07 Hz, 1 H) 8.19 ppm (s, 1 H).

(*R*)-3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)-1-(4-((5-methoxypyridin-2-yl)methyl)-2-methylpiperazin-1-yl)propan-1-one (9a): A solution of compound **7** (90 mg, 0.19 mmol) and Pt/C (5%) (32 mg, 8.20 µmol) in EtOH (4.7 mL) was stirred at RT under hydrogen for 21 h. The reaction mixture was filtered over Celite and concentrated to give 78 mg as a yellow oil. The crude product was purified by reversed-phase preparative HPLC (method C) to give 29 mg of pale yellow oil. The residue was diluted with CH_2Cl_2 and passed through a phase separator, concentrated and dried on high vacuum, at 50°C overnight to provide 24 mg of pale yellow oil (21%). LC/MS: t_{RB}=2.84 min, m/z: 485.0 [M+H]. ^{1}H NMR (400 MHz, DMSO) δ= 1.24 (d, *J* = 6.78 Hz, 4 H), 2.46 (d, *J* = 1.76 Hz, 3 H), 2.55 - 2.69 (m, 3 H), 2.90 - 3.05 (m, 3 H), 3.12 - 3.27 (m, 2 H), 3.89 (s, 3 H), 3.93 - 4.17 (m, 3 H), 4.47 (br. s., 1 H), 5.92 (s, 2 H), 7.20 - 7.42 (m, 3 H), 7.46 (s, 2 H), 8.32 ppm (s, 1 H).

(*R*)-1-(4-((5-methoxypyridin-2-yl)methyl)-2-methylpiperazin-1-yl)-3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl) propan-1-one (9b): A solution of compound **7** (24 mg, 0.05 mmol) and Pd/C (10%) (2.8 mg, 2.61 µmol) in EtOH (1.2 mL) was stirred at RT under hydrogen for 17h. The reaction mixture was filtered over Celite and concentrated to give 20.5 mg of a pale yellow oil (84%). LC/MS: t_{RB}=3.12 min, m/z: 518.0 [M+H]. ^{1}H NMR (400 MHz, DMSO) δ= 1.10 - 1.25 (m, 4 H), 1.78 - 2.08 (m, 2 H), 2.37 - 2.45 (m, 4 H), 2.56 - 3.20 (m, 6 H), 3.40 - 3.62 (m, 3 H), 3.82 (s, 3 H), 3.89 - 4.60 (m, 2 H), 6.06 (br. s., 2 H), 7.38 (s, 2 H), 7.55 (br. s., 1 H), 7.68 (br. s., 2 H), 8.19 ppm (s, 1 H).

5-(2-fluoroethoxy)-2-methylpyridine (10a): A suspension of 6-methylpyridin-3-ol (0.5 g, 4.58 mmol), 2-fluoroethyl-4-methylbenzenesulfonate (1.5 g, 6.87 mmol), K_2CO_3 (0.82 g, 5.96 mmol) and NaI (0.04 g, 0.26 mmol) was stirred at 80°C for 16 h under argon. The reaction mixture was allowed to cool to room temperature, diluted with EtOAc and washed with water three times. The combined aqueous layers were extracted with EtOAc. The combined organic layers were washed with brine, dried over a phase separator and concentrated. The crude product was purified by flash chromatography on silica gel (mobile phase: cyclohexane (A) / EtOAc (B); gradient: 100% A to 100% B in 15min), to afford 591 mg of an orange solid (83%). LC/MS: t_{RA}=0.37 min, m/z: 156.0 [M+H]. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 2.43 (s, 3 H), 4.07 - 4.16 (m, 1 H), 4.17 - 4.26 (m, 1 H), 4.56 - 4.68 (m, 1 H), 4.71 - 4.80 (m, 1 H), 6.97 - 7.03 (m, 1 H), 7.05 - 7.14 (m, 1 H), 8.15 ppm (d, *J* = 2.81 Hz, 1 H).

2-(2-((6-methylpyridin-3-yl)oxy)ethoxy)ethanol: A suspension of 6-methylpyridin-3-ol (1.5 g, 13.75 mmol), diethylene glycol monochlorohydrine (5.83 mL, 55.0 mmol), K_2CO_3 (2.85 g, 20.62 mmol) and NaI (0.11 g, 0.76 mmol) in DMF (18.6 mL) was stirred at 85°C for 16 h under an argon atmosphere. The reaction mixture was allowed to cool to room temperature, diluted with EtOAc and washed twice with water. The aqueous layer was extracted with CH_2Cl_2/i-PrOH 4:1. The combined organic layers were washed with brine, dried over a phase separator and concentrated. The combined organic layers were dried and concentrated to give the crude product as purple oil, which was purified by flash chromatography (mobile phase: CH_2Cl_2 (A) /MeOH (B); gradient: 0 to 10% B) to afford 5.0 g of brown oil, which was directly used in the next step. LC/MS: t_{RA}=0.32 min, m/z: 198.1 [M+H].

2-(2-((6-methylpyridin-3-yl)oxy)ethoxy)ethyl 4-methylbenzene sulfonate: To a stirred solution of 2-(2-((6-methylpyridin-3-yl)oxy)ethoxy)ethanol (2.35 g, 4.05 mmol) in CH_2Cl_2 (10 mL) was slowly added p-tolylsulfonyl chloride (1.87 g, 9.72 mmol) followed by triethylamine (2.82 mL, 20.26 mmol) at 0°C, under argon. The reaction mixture was stirred at RT for 16 h, then diluted with CH_2Cl_2 and water. The organic layer was washed with brine, dried over a phase separator and concentrated to give the crude product as brown oil, which was purified by flash chromatography (mobile phase: cyclohexane (A) / EtOAc (B); gradient: 100% A to 100% B) to afford 1.02 g of the desired product as yellow oil, and which was directly used in the next step. LC/MS: t_{RA}=0.85 min, m/z: 352.4 [M+H].

5-(2-(2-fluoroethoxy)ethoxy)-2-methylpyridine (10b): To a solution of 2-(2-((6-methylpyridin-3-yl)oxy)ethoxy)ethyl 4-methylbenzenesulfonate (1.02 g, 2.48 mmol) in THF (15.7 mL) was added TBAF (1M in THF, 12.40 mL, 12.40 mmol) and the resulting solution was stirred at 65°C for 30 min. The solvent was evaporated, and the residue taken up in EtOAc and washed with water. The aqueous layer was extracted twice with EtOAc. The combined organic layers were washed with brine, dried over a phase separator and concentrated to the give crude product as brown oil, which was purified by flash chromatography (cyclohexane (A) / EtOAc (B); gradient: 100% A to 100% B) to afford the title compound as orange oil (465 mg, 85%). LC/MS: t_{RA}=0.44 min, m/z: 200.1 [M+H]. ^{1}H NMR (400 MHz, DMSO): δ=2.41 (s, 3H), 3.64 - 3.72 (m, 1H), 3.76 - 3.83 (m, 3H), 4.16 (dd, *J* = 5.44, 3.73 Hz, 2H), 4.45 - 4.53 (m, 1H), 4.61 - 4.64 (m, 1H), 7.18 (d, *J* = 8.56 Hz, 1H), 7.31 (dd, *J* = 8.50, 3.00 Hz, 1H), 8.17 ppm (d, *J* = 2.93 Hz, 1H).

5-(2-fluoroethoxy)-2-methylpyridine 1-oxide: A solution of compound **10a** (591 mg, 3.81 mmol) in $CHCl_3$ (17.3 mL) at 0°C was added *m*-chloroperbenzoic acid (789 mg, 4.57 mmol). The resulting mixture was stirred at 0°C for 2 h. The reaction mixture was quenched with a saturated solution of Na_2CO_3 and extracted twice with CH_2Cl_2. The combined organic layers were washed with brine, dried over a phase separator and concentrated to give 145 mg of crude product as white solid, and which was used in the next step without further purification. LC/MS: t_{RA}=0.43 min, m/z: 172.0 [M+H]..

(5-(2-fluoroethoxy)pyridin-2-yl)methyl acetate (11a): To acetic anhydride (748 µl, 7.93 mmol) was added at 80°C 5-(2-fluoroethoxy)-2-methylpyridine 1-oxide (145 mg, 0.61 mmol). The reaction mixture was heated up to 130°C and stirred for 30 min., poured into ice water and then stirred at room temperature for 15 min. The product was extracted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$ and brine. The aqueous layer was extracted with EtOAc. The combined organic layers were dried over a phase separator and concentrated to give the crude product as brown oil, and which was purified by flash chromatography (mobile phase: cyclohexane (A) / EtOAc (B); gradient: 100% A to 40% B) to afford 73 mg of the title compound as yellow oil, and which was used in the next step without further purification. LC/MS: t_{RA}=0.66 min, m/z: 214.0 [M+H].

5-(2-(2-fluoroethoxy)ethoxy)-2-methylpyridine 1-oxide: To a solution of compound **10b** (465 mg, 2.1 mmol) in $CHCl_3$ (10.6 mL) at 0°C was added *m*-chloroperbenzoic acid (435 mg, 2.52 mmol). The resulting mixture was stirred at 0°C for 3 h, then quenched with sat. aq. Na_2CO_3 and extracted twice with CH_2Cl_2. The combined organic layers were washed with brine, dried over a phase separator and concentrated to give 562 mg of crude product as yellow oil, and which was used in the next step without further purification. LC/MS: t_{RA}=0.48 min, m/z: 216.1 [M+H].

(5-(2-(2-fluoroethoxy)ethoxy)pyridin-2-yl)methyl acetate (11b): To acetic anhydride (2018 µl, 21.39 mmol) was added at 80°C 5-(2-(2-fluoroethoxy)ethoxy)-2-methylpyridine 1-oxide (562 mg, 1.64 mmol). The reaction mixture was heated up to 130°C and stirred for 30 min., then poured into ice water and stirred at RT for 15 min. The product was extracted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$ and brine. The aqueous layer was extracted with EtOAc. The combined organic layers were dried over a phase separator and concentrated to give the crude product as a brown oil which was purified by flash chromatography (mobile phase: cyclohexane (A) / EtOAc (B); gradient: 100% A to 40% B), to afford 352 mg of title compound as yellow oil, and which was used in the next step without further purification. LC/MS: t_{RA}=0.71 min, m/z: 258.1 [M+H].

(5-(2-fluoroethoxy)pyridin-2-yl)methanol: To a solution of compound **11a** (430 mg, 1.81 mmol) in EtOH (4.7 mL) and water (0.82 mL) was added NaOH (145 mg, 3.63 mmol). The resulting reaction mixture was stirred at reflux for 1h. The solvents were evaporated and the residue extracted twice with EtOAc. The combined organic layers were dried over a phase separator and concentrated to give 340 mg of crude product as yellow oil, which was directly used in the next step. LC/MS: t_{RA}=0.44 min, m/z: 215.8 [M+H].

5-(2-fluoroethoxy)picolinaldehyde (12a): To a solution of (5-(2-fluoroethoxy)pyridin-2-yl)methanol (340 mg, 1.63 mmol) in CH_2Cl_2 (6.5 mL) was added MnO_2 (1416 mg, 16.29 mmol). The resulting reaction mixture was stirred at RT for 2 days. The reaction mixture was filtered over Celite and washed three times with EtOAc. The filtrate was evaporated to dryness to give 196 mg of crude product as yellow oil which was directly used in the next step. LC/MS: t_{RA}=0.61 min, m/z: 170.0 [M+H].

(5-(2-(2-fluoroethoxy)ethoxy)pyridin-2-yl)methanol: To a solution of compound **11b** (352 mg, 1.08 mmol) in EtOH (4.7 mL) and water (0.82 mL) was added NaOH (86 mg, 2.16 mmol). The resulting reaction mixture was stirred at reflux for 1h. The solvents were evaporated and the residue was extracted twice with EtOAc. The combined organic layers were dried over a phase separator and concentrated to give 282 mg of crude product as orange oil, which was directly used in the next step. LC/MS: t_{RA}=0.43 min, m/z: 216.1 [M+H].

5-(2-(2-fluoroethoxy)ethoxy)picolinaldehyde (12b): To a solution of (5-(2-(2-fluoroethoxy)ethoxy)pyridin-2-yl)methanol (282 mg, 1.05 mmol) in CH_2Cl_2 (4.0 mL) was added MnO_2 (911 mg, 10.5 mmol). The resulting reaction mixture was stirred at RT for 2 days, then filtered over Celite and washed three times with EtOAc. The filtrate was evaporated to dryness to give 185 mg of crude product as yellow oil, which was directly used in the next step. LC/MS: t_{RA}=0.65 min, m/z: 214.0 [M+H].

(*R*,*E*)-3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)-1-(4-((5-(2-fluoroethoxy)pyridin-2-yl)methyl)-2-methylpiperazin-1-yl)prop-2-en-1-one (2): To a solution of **6a** (100 mg, 0.28 mmol) in MeOH (2.5 mL) were added AcOH (0.25 mL) and **12a** (70.3 mg, 0.42 mmol). After stirring for 5 min, 2-picoline borane (55.3 mg, 0.44 mmol) was added. The reaction mixture was stirred at room temperature for 16 h. The volatiles were evaporated and the residue purified by reversed-phase preparative HPLC (method C) to provide a white solid (33 mg, 23%). LC/MS: t_{RA}=0.92 min, m/z: 548.0 [M+H]. ^{1}H NMR (400 MHz, DMSO, 100°C) δ=1.29 (d, *J* = 6.78 Hz, 3H) 2.00 - 2.14 (m, 1H) 2.21 - 2.29 (m, 1H) 2.44 (s, 3H) 2.66 - 2.76 (m, 1H) 2.84 - 2.92 (m, 1H) 3.12 - 3.25 (m, 1H) 3.60 (d, *J* = 10.67 Hz, 2H) 4.06 - 4.17 (m, 1H) 4.28 - 4.34 (m, 1H) 4.35 - 4.42 (m, 1H) 4.45 - 4.58 (m, 1H) 4.66 - 4.74 (m, 1H) 4.78 - 4.87 (m, 1H) 6.06 (s, 2H) 7.11 (d, *J* = 15.43 Hz, 1H) 7.42 (d, *J* = 1.88 Hz, 2H) 7.77 (d, *J* = 5.65 Hz, 3H) 7.92 - 8.04 (m, 1H) 8.26 ppm (s, 1H).

(*R*,*E*)-1-(4-((5-(2-fluoroethoxy)pyridin-2-yl)methyl)-2-methylpiperazin-1-yl)-3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)prop-2-en-1-one (13): To a solution of **6b** (100 mg, 0.25 mmol) in MeOH (2.3 mL) were added AcOH (0.23 mL) and **12a** (64.3 mg, 0.38 mmol). After stirring for 5 min, 2-picoline borane (50.6 mg, 0.41 mmol) was added. The reaction mixture was stirred at room temperature for 16 h. The volatiles were evaporated and the residue purified by flash chromatography to provide a white solid (81 mg, 58%). LC/MS: t_{RB}= 3.4 min, m/z: 548.1 [M+H]. ^{1}H NMR (400 MHz, DMSO, 100°C) δ=1.29 (d, *J* = 6.78 Hz, 3 H), 2.00 - 2.14 (m, 1 H), 2.21 - 2.29 (m, 1 H), 2.44 (s, 3 H), 2.66 - 2.76 (m, 1 H), 2.84 - 2.92 (m, 1H), 3.12 - 3.25 (m, 1 H), 3.60 (d, *J* = 10.67 Hz, 2 H), 4.06 - 4.17 (m, 1 H), 4.28 - 4.34 (m, 1 H), 4.35 - 4.42 (m, 1 H), 4.45 - 4.58 (m, 1 H), 4.66 - 4.74 (m, 1 H), 4.78 - 4.87 (m, 1 H), 6.06 (s, 2 H), 7.11 (d, *J* = 15.43 Hz, 1 H), 7.42 (d, *J* = 1.88 Hz, 2 H), 7.77 (d, *J* = 5.65 Hz, 3 H), 7.92 - 8.04 (m, 1 H), 8.26 ppm (s, 1 H).

(*R*,*E*)-3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)-1-(4-((5-(2-(2-fluoroethoxy)ethoxy)pyridin-2-yl)methyl)-2-methylpiperazin-1-yl)prop-2-en-1-one (14a): To a solution of **6a** (80 mg, 0.22 mmol) in MeOH (2.0 mL) were added AcOH (0.2 mL) and **12b** (93 mg, 0.33 mmol). After stirring for 5 min, 2-picoline borane (44.2 mg, 0.35 mmol) was added. The reaction mixture was stirred at RT for 20h. The volatiles were evaporated and the residue purified by preparative HPLC (method C). The desired fractions were concentrated. The residue was taken up in EtOAc and washed with sat. aq. $NaHCO_3$. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over a phase separator and concentrated to give 63 mg yellow oil (50%). LC/MS: t_{RA}=0.87 min, m/z: 558.2 [M+H]. ^{1}H NMR (400 MHz, DMSO, 100°C) δ=1.28 (d, *J* = 6.78 Hz, 3H), 2.01 - 2.15 (m, 1H), 2.25 (dd, *J* = 11.48, 3.07 Hz, 1H), 2.44 (s, 3H), 2.73 (d, *J* = 11.42 Hz, 1H), 2.88 (d, *J* = 11.29 Hz, 1H), 3.17 (br. s., 1H), 3.52 - 3.66 (m, 2H), 3.70 - 3.76 (m, 1H), 3.78 - 3.88 (m, 3H), 4.12 (d, *J* = 12.55 Hz, 1H), 4.18 - 4.28 (m, 2H), 4.44 - 4.56 (m, 2H), 4.57 - 4.65 (m, 1H), 5.96 (s, 2H), 7.03 (d, *J* = 15.31 Hz, 1H), 7.40 (d, *J* = 1.76 Hz, 2H), 7.44 - 7.51 (m, 2H), 7.71 (d, *J* = 15.31 Hz, 1H), 7.81 (d, *J* = 8.41 Hz, 1H), 8.24 ppm (t, *J* = 1.69 Hz, 1H).

(*R*,*E*)-1-(4-((5-(2-(2-fluoroethoxy)ethoxy)pyridin-2-yl)methyl)-2-methylpiperazin-1-yl)-3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)prop-2-en-1-one (14b): The title compound was prepared by a similar method to compound **14a**, from compounds **6b** and **12b** (85 mg, 0.30 mmol) to give 60 mg (49%) of title compound as yellow oil. LC/MS: t_{RA}=0.92 min, m/z: 592.2 [M+H]. ^{1}H NMR (400 MHz, DMSO) δ=1.29 (d, *J* = 6.65 Hz, 3 H) 2.09 (td, *J* = 11.67, 3.14 Hz, 1 H) 2.24 (dd, *J* = 11.36, 3.70 Hz, 1 H) 2.40 - 2.46 (m, 3 H) 2.72 (d, *J* = 11.29 Hz, 1 H) 2.88 (d, *J* = 11.17 Hz, 1 H) 3.12 - 3.27 (m, 1 H) 3.50 - 3.64 (m, 2 H) 3.70 - 3.75 (m, 1 H) 3.78 - 3.87 (m, 3 H) 4.12 (d, *J* = 13.80 Hz, 1 H) 4.19 - 4.26 (m, 2 H) 4.43 - 4.54 (m, 2 H) 4.57 - 4.65 (m, 1 H) 6.06 (s, 2 H) 7.11 (d, *J* = 15.43 Hz, 1 H) 7.40 (d, *J* = 1.13 Hz, 2 H) 7.69 - 7.84 (m, 3 H) 7.99 (d, *J* = 8.53 Hz, 1 H) 8.24 ppm (s, 1 H).

(*R*)-1-(4-((5-(2-fluoroethoxy)pyridin-2-yl)methyl)-2-methylpiperazin-1-yl)-3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)propan-1-one (15): A solution of compound **13** (46 mg, 0.084 mmol) and Pd/C (10%) (5.72 mg, 5.38 µmol) in EtOH (4.00 mL) was stirred at room temperature under hydrogen for 18 h. The reaction mixture was filtered over Celite and concentrated. The residue was taken up in 1mL of MeOH and purified by preparative HPLC (method C). The fractions containing the product were combined and lyophilized overnight. The resulting white powder was diluted with EtOAc and washed with a saturated solution of $NaHCO_3$. The aqueous layer was extracted with EtOAc and the combined organic layers washed with brine, dried over a phase separator, concentrated and dried under vacuum to give the title compound as yellow oil (22 mg, 47%). LC/MS: t_{RA}=0.89 min, m/z: 550.1 [M+H]. 2 min Final Analysis. ^{1}H NMR (400 MHz, DMSO) δ=1.16 - 1.22 (m, 3H), 1.27 - 1.31 (m, 1H), 1.92 - 2.13 (m, 2H), 2.46 (s, 3H), 2.55 - 2.85 (m, 4H), 3.04 - 3.07 (m, 3H), 3.48 - 3.62 (m, 2H), 3.78 - 4.03 (m, 1H), 4.28 - 4.41 (m, 2H), 4.66 - 4.85 (m, 2H), 5.96 - 6.08 (m, 2H), 7.35 - 7.44 (m, 2H), 7.53 - 7.71 (m, 3H), 8.21 - 8.29 ppm (m, 1H).

Methyl 2-(fluoromethyl)oxazole-4-carboxylate (16): To a solution of methyl 2-(chloromethyl)oxazole-4-carboxylate (1.0 g, 5.70 mmol) in CH_3CN (28.5 mL) was added TBAF (1M in THF, 17.09 mL, 17.09 mmol) at room temperature. The resulting green solution was stirred for 21 h under argon at RT. The reaction mixture was poured in water and extracted twice with EtOAc. The combined organic layers were washed with brine, dried over a phase separator and concentrated to give crude product as orange oil. The crude product was purified by preparative HPLC (method C). The fractions containing the desired product were combined, diluted with EtOAc and washed with sat. aq. $NaHCO_3$. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over a phase separator and concentrated to give 291 mg of a white solid (32%). LC/MS: t_{RA}=0.49 min, m/z: 160.0 [M+H]. ^{1}H NMR (400 MHz, DMSO): δ=3.76 (s, 3 H) 5.43 (s, 1 H) 5.55 (s, 1 H) 8.91 ppm (d, *J* = 1.34 Hz, 1 H).

(2-(fluoromethyl)oxazol-4-yl)methanol: To a solution of compound **16** (291 mg, 1.46 mmol) in THF (3.6 mL) at -78°C under argon, was added dropwise DIBAL-H (1M in THF, 3.22 mL, 3.22 mmol). The reaction mixture was then stirred at -78°C for 3 h and at room temperature for 16 h. UPLC/MS showed remaining starting material therefore the reaction mixture was cooled down to -78°C and DIBAL-H (1M in THF, 3.22 mL, 3.22 mmol) was added and the reaction mixture stirred at -78°C for 3 h, then diluted with CH_2Cl_2 at -78°C, quenched with MeOH/water/2M aq. NaOH (1 mL) and stirred for 15 min. Solid Na_2SO_4 was added at room temperature and the suspension stirred for 15 min. The salts were removed by filtration and the filtrate concentrated under vacuum. The crude product was purified by flash chromatography (mobile phase: CH_2Cl_2 (A) / MeOH (B); gradient: : from 100% A to 5% B), to afford the title compound as yellow oil (79 mg, 41%). LC/MS: t_{RA}=0.32 min, m/z: 132.0 [M+H]. ^{1}H NMR (400 MHz, DMSO) δ=4.31 - 4.34 (m, 2H) 5.32 (s, 1H) 5.44 (s, 1H) 7.84 - 8.03 ppm (m, 1H).

2-(fluoromethyl)oxazole-4-carbaldehyde (17): To a solution of (2-(fluoromethyl)oxazol-4-yl)methanol (78 mg, 0.6 mmol) in CH_2Cl_2 (6 mL) was added MnO_2 (517 mg, 5.95 mmol). The resulting mixture was stirred at RT for 2 days. The reaction mixture was filtered over Celite and washed three times with CH_2Cl_2. The filtrate was evaporated to dryness to give 32 mg of crude product, which was used in the next step without further purification. LC/MS: t_{RA}=0.34 min, m/z: 130.0 [M+H].

(*R*,*E*)-1-(4-((2-(fluoromethyl)oxazol-4-yl)methyl)-2-methylpiperazin-1-yl)-3-(2-((5-methyl-2H-tetrazol-2-yl)methyl)-4-(trifluoromethyl)phenyl)prop-2-en-1-one (18): The title compound was prepared by a similar method to compound **14a**, from compounds **6b** (80 mg, 0.20 mmol) and **17** (26.2 mg, 0.20 mmol), to give a of colourless oil (25 mg, 24%). LC/MS: t_{RA}=0.89 min, m/z: 508.3 [M+H]. ^{1}H NMR (400 MHz, DMSO) δ=1.27 (d, *J* = 6.97 Hz, 3H), 2.05-2.11 (m, 1H), 2.23-2.27 (m, 1H), 2.44 (s, 3H), 2.76-2.80 (m, 1H), 2.90-2.95 (m, 1H), 3.10-3.20 (m, 1H), 3.5 (s, 2H), 4.1 (d, 1H), 4.5 (s, 1H), 4.45 (d, 2H), 6.06 (s, 2H), 7.1 (d, 1H), 7.70 - 7.80 (m, 4H), 7.98-8.01 (m, 1H).

(*R*,*E*)-3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)-1-(4-((5-hydroxypyridin-2-yl)methyl)-2-methylpiperazin-1-yl)prop-2-en-1-one (19): A stirred solution of compound **6a** (3.0 g, 8.3 mmol) in $(ClCH_2)_2$ (60 mL) was treated with 5-hydroxypyridine-2-carboxaldehyde (2.0 g, 16.6 mmol), sodium triacetoxyborohydride (3.5 g, 16.62 mmol) and acetic acid (0.95 mL) at 0°C. The resulting mixture was stirred at room temperature for 17 h. The solvent was evaporated, water was added to the residue, and the aqueous phase extracted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$, brine, dried over solid Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by flash chromatography on silica gel (mobile phase: CH_2Cl_2 (A) / MeOH (B); gradient: from 100% A to 5% B), to afford the title compound as a white powder (2.8 g, 72%). LC/MS: t_{RA}= 0.73 min, m/z: 468.2 [M+H]. ^{1}H NMR (600 MHz, DMSO-d6) δ=1.24 (s, 3H), 2.19-2.89 (m, 2H), 2.39 (s, 1H), 2.41 (s, 3H), 2.62 (s, 1H), 2.66 (d, *J* = 11.3 Hz, 1H), 2.82 (s, 1H), 3.45 (d, *J* = 13.7 Hz, 1H), 3.53 (d, *J* = 13.7 Hz, 1H), 3.98-4.28 (m, 1H), 4.36-4.65 (m, 1H), 5.96-6.05 (m, 2H), 7.11-7.19 (m, 1H), 7.28 (d, *J* = 8.4 Hz, 1H), 7.51-7.53 (m, 1H), 7.72 (d, *J* = 15.2 Hz, 1H), 7.90 (d, *J* = 8.6 Hz, 1H), 8.04 (d, *J* = 2.8 Hz, 1H), 9.78 (s, 1H).

(*R*,*E*)-2-((6-((4-(3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl)phenyl)acryloyl)-3-methylpiperazin-1-yl)methyl)pyridin-3-yl)oxy)ethyl 4-methylbenzenesulfonate (20) : Cs_2CO_3 (446 mg, 1.37 mmol) was added to a solution of ethylene di(p-toluenesulfonate) (475 mg, 1.28 mmol) in DMF (20 mL) under argon at room temperature, followed by the addition of compound **19** (200 mg, 0.43 mmol) in DMF (20 mL) over a period of 1 h. The reaction mixture was stirred at room temperature for 2 h, then diluted with water. The aqueous phase was extracted with ethyl acetate. The combined organic phases were dried (Phase Separator) and concentrated. The crude product was purified by flash chromatography on silica gel (mobile phase: cyclohexane (A) / EtOAc (B); gradient: 100% A to 100% B), to obtain the product as colourless oil (180 mg, 62%). LC/MS: t_{RA}=1.06 min, m/z: 666.5 [M+H]. ^{1}H NMR (400 MHz, 300°C, DMSO) δ=1.12-1.27 (m, 3H), 1.97 (s, 1H), 2.11 (s, 1H), 2.40 (d, *J* = 4.0 Hz, 6H), 2.64 (d, *J* = 11.3 Hz, 1H) 2.80 (d, *J* = 11.0 Hz, 1H), 3.27 (s, 1H), 3.44-3.59 (m, 2H), 4.01 (q, *J* = 7.1 Hz, 1H), 4.22 (dd, *J* = 5.3, 2.8 Hz, 2H), 4.34 (dd, *J* = 5.3, 2.8 Hz, 2H), 4.4 (s, 1H), 6.03-5.91 (m, 2H), 7.11 (d, *J* = 15.2 Hz, 1H), 7.29 (dd, *J* = 8.6, 2.9 Hz, 1H), 7.35 (d, *J* = 8.6 Hz, 1H), 7.43-7.51 (m, 4H), 7.70 (d, *J* = 15.2 Hz, 1H), 7.78 (d, *J* = 8.3 Hz, 2H), 7.87 (d, *J* = 9.1 Hz, 1H), 8.07 ppm (d, *J* = 2.8 Hz, 1H).

[^{18}F]-(*R*,*E*)-3-(4-chloro-2-((5-methyl-2H-tetrazol-2-yl)methyl) phenyl)-1-(4-((5-(2-fluoroethoxy)pyridin-2-yl)methyl)-2-methylpiperazin-1-yl)prop-2-en-1-one ([^{18}F]2): [^{18}F]fluoride produced via the ^{18}O(p,n)^{18}F nuclear reaction was purchased from PETNET. The [^{18}F]fluoride in 1.5 mL of [^{18}O]H2O was trapped on a QMA-Light Sep-Pak (Waters Accell Plus QMA cartridge, 46 mg, PN 1860045-40). It was then eluted into the reactor with a solution of K_2CO_3 (0.75 mg) and Kryptofix-2.2.2 (7.5 mg) in deionized water (0.4 mL) and MeCN (0.4 mL). The [^{18}F]fluoride was dried by heating under vacuum (5 min. at 70 °C and 5 min. at 100 °C) in a stream of nitrogen. A solution of **20** (2 mg, 3 μmol) in anhydrous. DMSO (0.7 mL) was added and heated to 140 °C under stirring for 10 min., then cooled to 50 °C, diluted with 4.3 mL of HPLC water, and the mixture passed through an Alumina N Light cartridge. The filtrate was loaded onto a semi-preparative HPLC system for purification (column: Phenomenex Luna C18(2), 5 μm, 250 x 10 mm, CAT# 00G-4252-N0; mobile phase: 50% MeCN /50% water/0.1% v/v TEA; flow: 4 mL-min). The product peak (rt = 12.2 min.) was collected into a flask containing 0.5% w/v NaAsc in water (45 mL). The purified [^{18}F]**2** was trapped on a C18-E cartridge (Phenomenex, C18-E-50 mg cartridge, PN 8B-S001-DAK), washed with 0.5% w/v NaAsc in water (5 mL), eluted with EtOH (0.5 mL), then diluted with 0.33% w/v NaAsc, 0.33% v/v Tween-80 in 0.9% w/v NaCl (4.5 mL). The [^{18}F]**2** formulation was transferred to a sterile dose vial and submitted for quality control. The radiochemical yield was ~15% (decay corrected) and produced a tracer with <99% radiochemical purity, >93% chemical purity and a specific activity of 304±136 GBq/μmole.

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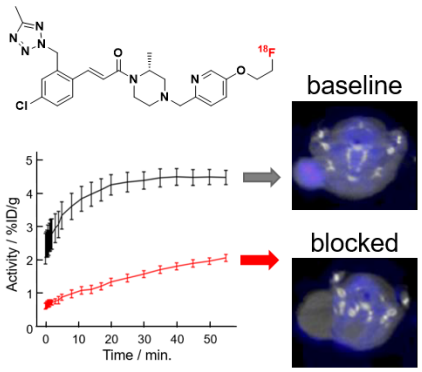

PRIMATX is a novel PET tracer for imaging the secreted enzyme autotaxin (ATX). It proved sensitive to fibrosis-associated ATX expression in fibrotic lung tissue from idiopathic pulmonary fibrosis patients, and allows the detection of autotaxin-expressing melanoma tumors in living mice with a good signal-to-noise ratio. This study provides a potential diagnostic tool for clinical development, and confirms the relevance of ATX for this purpose.