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Letter

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Synthesis and preclinical evaluation of 6-[^{18}F]Fluorine-Alpha-Methyl-L-Tryptophan, a novel PET tracer for measuring tryptophan uptake.

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ABSTRACT: The Positron emission tomography (PET) radioligand α-[^{11}C]Methyl-L-tryptophan ([^{11}C]AMT) has been used to assess tryptophan metabolism in cancer, epilepsy, migraine and autism. Despite the extensive application, the utility of this tracer is currently hampered by the short half-life of the radionuclide used for its labeling (^{11}C, $t_{1/2}$ = 20.4 min). We herein report the design, synthesis, radiolabeling and initial *in vivo* evaluation of a fluorine-18 (^{18}F, $t_{1/2}$ = 109.7 min) labeled analog that is fluorinated in the 6-position of the aromatic ring ([^{18}F]6-F-AMTr). In a head-to-head comparison between [^{18}F]6-F-AMTr and [^{11}C]AMT in mice using PET, peak brain radioactivity, regional brain distribution and kinetic profiles were similar between the two tracers. [^{18}F]6-F-AMTr was however not a substrate for IDO1 nor TPH as determined in *in vitro* enzymatic assays. The brain uptake of the tracer is thus more likely related to LAT1 transport over the blood-brain barrier than metabolism along the serotonin or kynurenine pathways.

α-[^{11}C]Methyl-L-tryptophan ([^{11}C]AMT) is a positron emission tomography (PET) tracer originally designed to measure the metabolism of tryptophan into serotonin in brain.[1] However, because of the extensive oxidative metabolism of tryptophan along the kynurenine pathway, which may represent up to 95% of tryptophan turnover,[2] the uptake of [^{11}C]AMT is more likely to reflect activity along the kynurenine pathway during disease states where this pathway is induced.[3-5] During recent years, immune activation has been identified as a key factor that can induce the kynurenine pathway, in particular in relation to cancer where inhibitors of indoleamine 2,3-dioxygenase (IDO), the initial key enzyme of the pathway, have been developed as a putative therapeutic approach.[6] [^{11}C]AMT, which is a substrate of IDO, has consequently shown increased uptake in brain, breast and lung tumours.[4, 7, 8] [^{11}C]AMT has also found utility as a clinical research tool in other disease conditions, including epilepsy, migraine and tuberous sclerosis.[9-11]

The kynurenine pathway has been suggested as a putative disease mechanism also in psychiatric disorders. [12, 13] Specifically, immune-related induction of the kynurenine pathway has been shown to increase endogenous brain levels of kynurenic acid (KYNA), an antagonist of glutamatergic N-methyl-D-aspartate receptors (NMDAR).[12] KYNA has been found to be robustly increased in the CNS of patients with schizophrenia, a disorder where NMDAR antagonism is an important candidate disease mechanism. Similarly, depression and suicidal behavior is associated with induction of the kynurenine pathway and increased production of quinolinic acid (QUIN), an NMDAR agonist.[13, 14] Interestingly, aberrant brain uptake of [^{11}C]AMT has been associated with depressive symptoms in patients with brain tumours,[4] and altered uptake has been reported in autism,[15] indicating its potential utility as an imaging biomarker for psychiatric conditions.

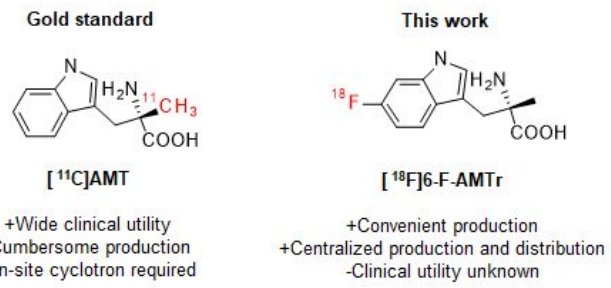
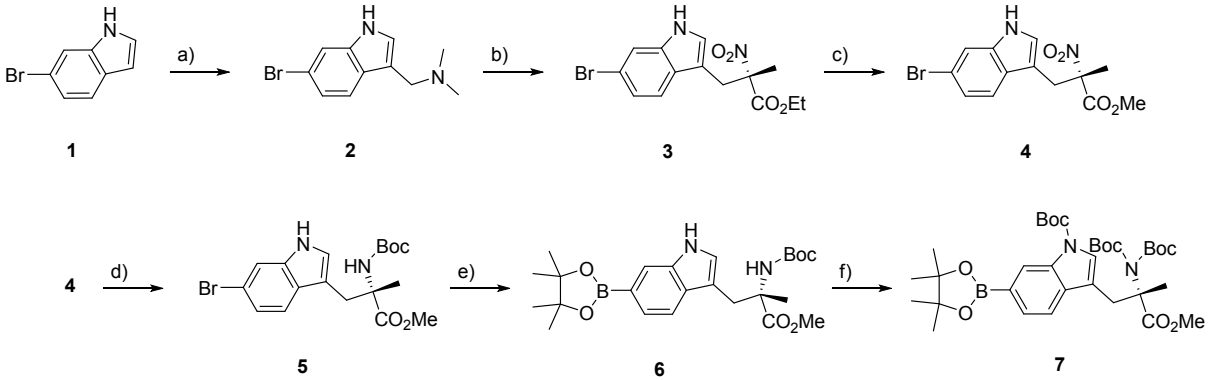


Figure 1. A comparison of some inherent properties of [^{11}C]AMT, and its proposed radiofluorinated analog, [^{18}F]6-F-AMTr.

One limitation of [^{11}C]AMT is the short half-life of ^{11}C ($t_{1/2}$ = 20.4 min). In an effort to provide wide access to AMT PET imaging in a clinical setting we aimed to design and label an analog of AMT with fluorine-18, a radionuclide that has several inherent advantages compared to carbon-11. Most notably, its half-life of 110 min allows for more complex synthesis and transportation to PET centers that lack an on-site cyclotron. Indeed, several radiolabeled tryptophan derivatives have been reported in the literature.[16-22] Among these, a commonly adopted strategy in tracer design has been to introduce an aliphatic side-chain to enable facile radiolabeling. In this work, it was hypothesized that direct fluorination of the aromatic moiety would be most favorable for retention of the biological and physicochemical properties of AMT, since it merely involves the substitution of a hydrogen with a fluorine atom. We here report the synthesis and radiolabeling of an analog of AMT that is fluorinated in the 6-position of the aromatic ring 6-[^{18}F]fluoro-α-methyl tryptophan ([^{18}F]6-F-AMTr, Fig. 1) and its in vivo evaluation and comparison with [^{11}C]AMT in mice.

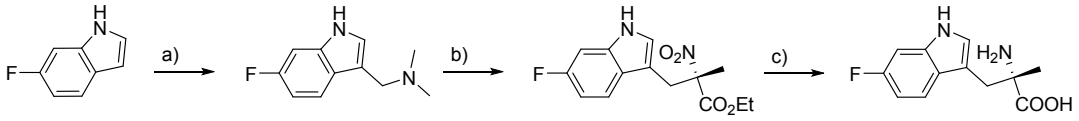
The synthesis of enantiomerically pure pinacol-borane precursor **7 (Scheme 1)** was achieved in 6 steps starting from 6-bromo indole (**1**). Briefly, **1** was reacted with formaldehyde and dimethylamine in acetic acid under Mannich reaction conditions to give the corresponding alkylated indole **2**. Treatment of **2** with ethyl 2-nitropropanoate in refluxing toluene afforded racemic **3**, which was purified by chiral HPLC to yield the pure (*S*)-enantiomer. Trans-esterification of (*S*)-**3** under alkaline conditions yielded methyl ester (*S*)-**4**, which was reduced to the corresponding protected amino acid **5** by catalytic hydrogenation in the presence of Raney Nickel. Under Miyaura borylation conditions compound **6** was formed. The final compound **7** was obtained after double *N*-Boc protection of the both nitrogens using Boc anhydride under DMAP activation. Enantiomerically pure 6-F-AMTr was prepared in 4 synthetic steps from commercially available 6-fluoro-indole in an overall yield of 7.5% (see Scheme 2). While this work was in progress, Giglio et al. reported on the synthesis and in vivo evaluation of another fluorinated AMT analog, namely 5-[^{18}F]fluoro-AMT.[18] A similar protocol was employed for both precursor and reference material synthesis. Nevertheless, the procedure developed for this study provided the target compounds at high chemical and enantiomeric purity suitable for the intended preclinical PET study.

The radiosynthesis of [^{18}F]6-F-AMTr was accomplished via a one-pot three-step synthesis (Scheme 3). First, ^{18}F-fluoride was incorporated under copper-mediated fluorination conditions.[21, 23, 24] The radiochemical conversion (RCC) was 41% (based on radio-TLC analysis of the reaction mixture). Following acidic deprotection of *N*-Boc protecting groups and saponification, [^{18}F]6-F-AMTr was purified using semi-preparative HPLC, isolated using mixed mode solid phase extraction, and formulated in a solution appropriate for intravenous injection. The overall isolated radiochemical yield of [^{18}F]6-F-AMTr was 7.5% (unoptimized, uncorrected for decay) and the radiochemical purity was >97% (determined by analytical radio-HPLC). The molar activity was 58 GBq/μmol at time of administration, corresponding to 1 μg of administered 6-F-AMTr. Thus, a radiochemical synthesis was developed that provided the target tracer in sufficient radioactivity, at high radiochemical purity and at high molar activity to enable a preclinical evaluation in vivo in mice. Although the overall isolated radiochemical yield was moderate, it was envisaged that this could be improved substantially if the tracer were translated to imaging in human subjects, in particular given the efficient incorporation of ^{18}F-fluoride in the radiolabeling step.



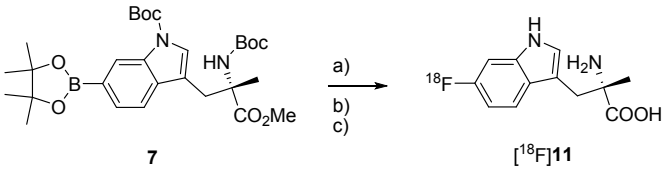
Scheme 1. Synthesis of aryl boronic ester precursor for radiolabeling of 6-F-AMTr with fluorine-18. Reagents and conditions: a) HCHO, Me_2NH, AcOH, 0 °C-RT. b) ethyl 2-nitropropanoate, toluene, reflux followed by chiral separation c) MeONa, MeOH, RT. d)

Raney Ni, H_2 (balloon), MeOH, Boc2O, RT. e) Bis(pinacolato)diboron, $Pd(dppf)Cl_2$, KOAc, DME. μwave, 105 °C f) Boc_2O, DMAP, TEA, MeCN. RT.



8 9 10 11

Scheme 2. Synthesis of 6-F-AMTr. Reagents and conditions: a) HCHO, Me_2NH, AcOH, 0 °C-RT. b) ethyl 2-nitropropanoate, toluene, reflux followed by chiral separation c) i) Raney Ni, H_2 (balloon) MeOH, RT, ii) LiOH, THF/H_2O (1:1), RT.



7 [^{18}F]**11**

Scheme 3. Radiolabeling of 6-F-AMTr with fluorine-18. Reagents and conditions: a) [^{18}F]Et_4NF, $Cu(OTf)_2{\cdot}Py_4$, DMF/MeCN, 110 °C. b) HCl, 100 °C c) NaOH, 110 °C

Human L-type amino acid transporter 1 (LAT1) is a transmembrane protein capable of facilitating transport of large hydrophobic/aromatic amino acids such as leucine, tyrosine, and tryptophan etc. across membranes with broad specificity.[25] Since [^{18}F]6-F-AMTr is a close derivative of tryptophan we hypothesized that LAT1 would have a similar activity towards this novel tracer. To test this hypothesis, we studied the docking of 6-F-AMTr, tryptophan and several other compounds with known inhibitor activity towards LAT1 in an inward open conformation. To the best of our knowledge, a detailed study of the interaction with LAT1 has not been performed yet for [^{11}C]AMT either. Results of the docking are shown in Fig.2 and Table 1S (see supporting information). A significant correlation (R=0.74) between ICM docking scores and observed IC_{50}-values for the inhibition of Leu and His transport was observed.[26, 27] 6-F-AMTr showed similar or even better ICM-scores than related compounds, thus indicating its transport potential via LAT1.

Analysis of the LAT1 binding site in an inward open conformation (PDB: 6IRS) showed it is three times larger than required by Trp, the bulkiest natural aromatic amino acid. The inner surface of the binding site possesses a variety of hydrophilic and hydrophobic parts, thus providing a suitable binding environment for structurally diverse ligands. This is the most probable reason behind the observed promiscuity of LAT1. Our docking results also indicate that the ligands may have several docking poses with comparable docking scores (data not shown). It is noteworthy that the best score poses of Trp and its close analogues AMT and 6-F-AMTr in the LAT1 binding site are different due to steric clashes of the alpha-methyl group with the Gly-255 backbone protruding into the binding site pocket (Fig.2). To eliminate this steric clash, the side chains of AMT and 6-F-AMTr adopt a different conformation while having a comparable ICM docking score. Notably 6-F-AMTr binding is ~3kcal/mol stronger than that of AMT due to the favorable interaction between F-atom and OH-group of Ser-342.

In the PET experiments, intravenous administration of either [^{11}C]AMT or [^{18}F]6-F-AMTr resulted in a brain exposure of around 100%SUV, suggesting that both tracers are indeed recognized by LAT1 (Fig 3,4). There were only small regional differences in brain radioactivity following tracer injection, with slightly higher radioactivity in the cerebellum and striatum than in other examined brain regions.

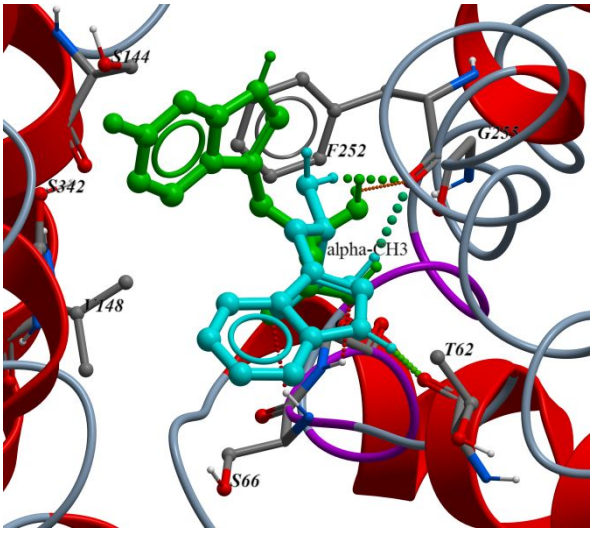


Figure 2. The lowest energy poses of tryptophan (magenta) and 6-F-AMTr (green) bound in active site of LAT1 transporter in the inwards open conformation (PDB code 6IRS).

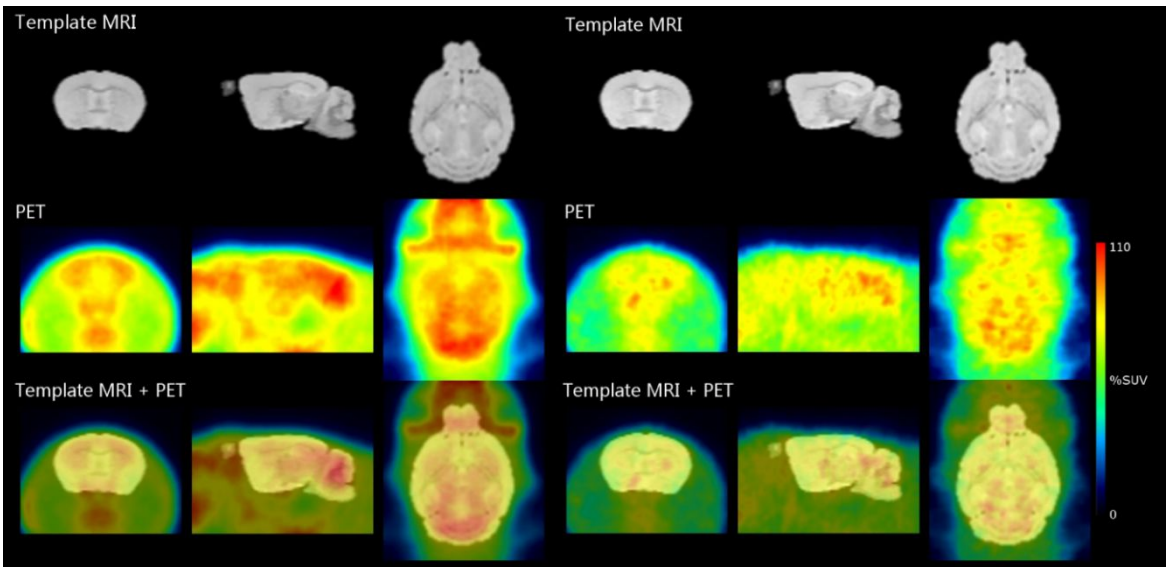
After extensive clinical validation, it has been postulated that the initial transport of [^{11}C]AMT over the BBB is followed by trapping of [^{11}C]AMT metabolites, formed either via the serotonin or the kynurenine pathways.[9] Thus, the shape of time activity curves (TACs) following administration of [^{11}C]AMT indicate irreversible uptake, i.e with a notable absence of washout from brain.[9] In this study, the average TACs obtained after intravenous injection of

[^{18}F]6-F-AMTr continuously increased for the duration of the PET measurement (93 min) while [^{11}C]AMT showed a slight plateau from 75 minutes. The uptake of [^{18}F]6-F-AMTr in the whole brain was between 1.1% (average) and 1.8% (peak value) of injected radioactivity while [^{11}C]AMT showed an uptake of 0.9% (average) and 1.4% (peak value). No apparent accumulation of radioactivity was observed in skull, indicating that only negligible (if any) defluorination of the radiotracer was taking place.. Interestingly, an analogous 6-[^{18}F]fluoro-tryptophan presented by Zlatopolskiy et al. demonstrated rapid in vivo defluorination, which indicates the importance of α-Me-group for metabolic stability of the tracer.[21] The mechanism for the trapping of [^{18}F]6-F-AMTr in brain remains elusive, however, it was not found to be a substrate for either IDO-1, nor TPH-1 or TPH-2 in enzymatic assays (See supporting information).

PET data was quantified by calculating the AUC (0-93 min) and the %SUV for the last hour of the experiment (33-93 min). The values for whole brain, striatum and cerebellum are shown in Figure 3. There was a small, but consistent, difference between the parameters with a 5-15% higher brain uptake for [^{18}F]6-F-AMTr compared to [^{11}C]AMT, depending on the region analyzed and the method used for the analysis. A possible explanation for both the slower kinetic profile and the higher brain exposure observed for [^{18}F]6-F-AMTr compared to [^{11}C]AMT could be that the metabolism and/or elimination of the former tracer is somewhat slower. This would result in a higher fraction of unchanged tracer in systemic circulation available for transportation across the BBB. However, a detailed study of the radiometabolites of [^{18}F]6-F-AMTr would be required to support this hypothesis. Another explanation is that [^{18}F]6-F-AMTr is more avidly transported by LAT1 across the BBB. This is supported by our computational docking study.

While this work was in progress, Giglio et al. reported the preparation and in vivo evaluation of an AMT analog that was radiofluorinated in the 5-position of the aromatic ring,[18] as opposed to the 6-position that was fluorinated in this study. In vitro data showed remarkable specificity for IDO, which demonstrated that delicate changes in the aromatic substitution pattern might have a great effect on the enzyme recognition of these fluorinated tracers. With regards to the in vivo evaluation of this 5-^{18}F-fluorinated tracer, only limited details were presented, although the PET images clearly showed localization of the tracer in tumor tissue. Since the fluorination position is expected to be important in relation to the in vivo disposition and metabolic trapping of AMT based tracers, a head- to head comparison of these two tracers *in vitro* and *in vivo* is highly warranted.

a



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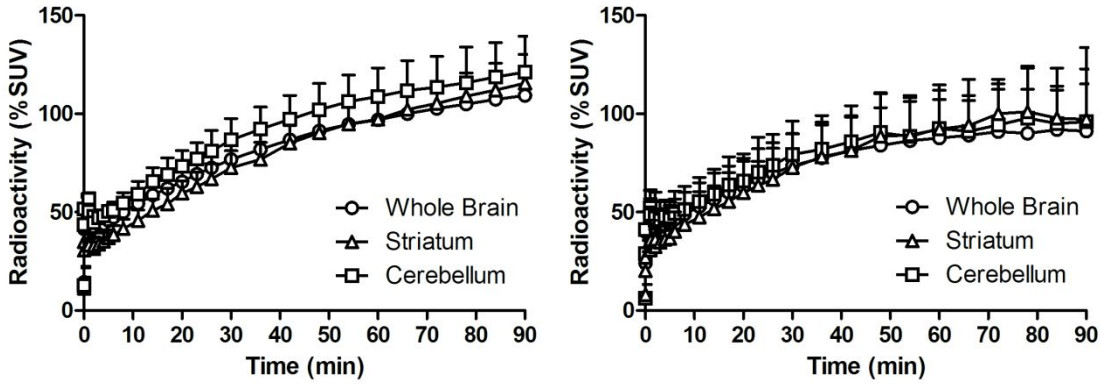

c

	AUC (0-93min)			%SUV (33-93min)		
	[^{18}F]6-F-AMTr	[^{11}C]AMT	% diff.	[^{18}F]6-F-AMTr	[^{11}C]AMT	% diff.
Whole Brain	7883 ± 805	7166 ± 1658	9.5%	97.6 ± 10.5	87.0 ± 20.4	11.5%
Striatum	7715 ± 827	7323 ± 1750	5.2%	98.8 ± 10.6	92.0 ± 22.2	7.2%
Cerebellum	8773 ± ^{11}14	7549 ± 1664	15.0%	108.7 ± 14.8	91.3 ± 20.2	17.4%

Figure 3. a) Average %SUV images following intravenous injection of [^{18}F]6-F-AMTr (left) and [^{11}C]AMT (right) (n = 4/group; 0-93 min). PET images for the coronal, sagittal and horizontal planes are shown, with the corresponding MRI template sections presented on top and the fused brain MRI and PET image below. **b)** Average TACs of whole brain, striatum and cerebellum in mice injected with [18F]6-F-AMTr (left) and [^{11}C]AMT (right) during the 93 min PET measurement (n = 4/group). **c**) [^{18}F]6-F-AMTr and [^{11}C]AMT AUC (0-93 min) and %SUV (33-93 min) values (mean ± SD) presented for whole brain, striatum and cerebellum (n = 4/group).

CONCLUSION

[^{18}F]6-F-AMTr, a novel alpha-methyl tryptophan analog, was prepared. It was stable towards defluorination in vivo and its brain exposure and kinetic profile was consistent with that observed for the extensively used [^{11}C]AMT. The tracer was however not a substrate for IDO-1 nor TPH1 or TPH2, and according to in silico docking studies, the brain uptake is more likely related to LAT1 transport than metabolism along the serotonin or kynurenine pathways.

METHODS

Preparation of [^{18}F]6-F-AMTr. A solution of [^{18}F]Fluoride ion in ^{18}O-enriched water was passed through a QMA-CO_3 SepPak light cartridge to isolate the [^{18}F]Fluoride ion. [^{18}F]Fluoride ion was eluted into the reaction vessel with 800 µL of a freshly prepared solution of tetraethyl ammonium bicarbonate ($TEAHCO_3$, 1 mg, 0.005 mmol) in anhydrous methanol (MeOH, 1 mL). The cartridge was rinsed with an additional 500 µL of anhydrous MeOH and the solution was collected into the same reaction vessel. The solvent was evaporated to dryness by heating at 100 °C under a stream of nitrogen (100 ml/min). To the dried [^{18}F]Et_4NF complex a solution of **7** (14.4 mg, 0.022 mmol) and $Cu(py)_4(OTf)_2$ (3.3 mg, 0.005 mmol) in a 10:1 mixture of anhydrous N,N-dimethyl formamide (DMF) and MeCN (550 µL) was added. The reaction mixture was heated at 110 °C for 20 min under air in the sealed 10 mL V-shaped reaction vial without stirring. The temperature was decreased to 40 °C and 250 µL of a 6M aqueous solution of HCl was added. The resulting mixture was heated for 10 min at 100 °C. After an additional cooling of the reaction mixture to 40 °C, 1 mL of a 5M aqueous solution of NaOH was added and the vessel was heated at 110 °C for 10 min. The reaction was quenched at room temperature by addition of a

few drops of TFA, prior to injection into a semi-preparative reverse phase HPLC ACE C-18 column and purified using 20:80 MeCN:TFA (0.1%) mixture as mobile phase. The fraction containing the desired compound was diluted in 50 mL of sterile water and loaded onto a Sep-Pak Oasis MCX cartridge. The cartridge was washed with 5 mL of water and, then, [^{18}F]6-F-AMTr was eluted from the cartridge using a 1 mL solution of aqueous NH_4OH (30%) in MeOH (5:95, v/v). Solvents were evaporated to dryness at 90 °C under nitrogen flow, after which the target tracer was reconstituted in a mixture of PBS and a solution of ethanol (30%) in propylene glycol (10:3, v/v mixture between PBS and propylene glycol solution).

ASSOCIATED CONTENT

SUPPORTING INFORMATION

Chemical synthesis of precursor and reference materials, enzyme assay procedures and further details on animal experiments.

AUTHOR INFORMATION

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict of Interest

Magnus Schou and Peter Johnström are employees and shareholders of AstraZeneca.

Notes

MS and PJ are employees and shareholders at AstraZeneca.

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