Tracer development for detection and characterization of neuroendocrine tumors with PET

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Chapter 6
5-Fluorotryptophan as potential PET tracer for neuroendocrine tumors

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Summary

[^{11}C]-5-HTP scanning in patients showed powerful results in the detection of neuroendocrine tumors. An ^{18}F labeled tryptophan analog with a longer radioactive half-life than [^{11}C]-5-HTP would increase clinical applicability because availability of [^{18}F]fluorotryptophan enables widespread use. Our results show that 5-fluorotryptophan (5-FTP) has, similar to 5-HTP, favorable characteristics to accumulate in a human neuroendocrine tumor cell line. An enzymatic synthesis towards 5-FTP gave high yields (73 ± 6 %) showing that an analogous synthesis of [^{18}F]-5-FTP is feasible. 5-FTP is a potential tracer for the detection of neuroendocrine tumors.

Introduction

Positron emission tomography (PET) of neuroendocrine tumors (NETs) demonstrated high contrast images by using the labeled large amino acids 6-[^{18}F]fluoro-levodopa ([^{18}F]FDOPA) and [^{11}C]-5-hydroxytryptophan ([^{11}C]-5-HTP). While [^{18}F]FDOPA is widely used in imaging NETs, the use of [^{11}C]-5-HTP is limited to centers with a cyclotron on site due to the short half-life of ^{11}C (20.4 min). To date, only a few PET-centers worldwide are performing successful studies with [^{11}C]-5-HTP PET for staging of neuroendocrine tumors[1-6]. Given the interesting results with [^{11}C]-5-HTP scanning in patients and the short half-life of [^{11}C]-5-HTP, the interest in an ^{18}F labeled tryptophan analog (half-life ^{18}F is 110 min) has increased in the past years[7,8].

Amine precursor uptake and decarboxylation (APUD)[9] in NET plays a major role in developing new amino acids for precise staging of NETs. Labeling of amino acids with ^{18}F has been described by using [^{18}F]fluoride as well as [^{18}F]F_2 gas[8]. The nucleophilic substitution reaction with [^{18}F]fluoride is usually the preferred labeling method because of its high specific radioactivity. However, direct ring labeling is not possible because these aromatic rings are not activated for nucleophilic substitution. The electrophilic substitution reaction with [^{18}F]fluorine gas is the most widely used method to label large amino acids containing an aromatic ring structure like [^{18}F]FDOPA directly at the ring position[10-14].

5-Fluorotryptophan (5-FTP) is an analogue of tryptophan with the characteristic properties of an amino acid. 5-FTP contains a fluorine atom that is not present in natural amino acids and therefore does not belong to the group of proteinogenic amino acids. To verify if 5-FTP is a potential PET tracer following the APUD principle we studied non-labeled tracer accumulation in a human NET cell line (BON) with 5-hydroxytryptophan (5-HTP) and 5-FTP using amino acid decarboxylase (AADC) and monoamine oxidase (MAO) inhibitors. AADC is responsible for the decarboxylation of amino acids such as 5-HTP and levodopa into their corresponding amines. MAO metabolizes these amines to 5-hydroxyindole acetic acid, respectively homovanillic acid. A tentative view on the metabolism of 5-FTP in neuroendocrine tumors is shown in figure 1.

For the synthesis of [^{18}F]-5-FTP our strategy was to combine methods used in the synthesis of [^{11}C]HTP[1] and [^{18}F]FDOPA[14]. The synthesis can be divided into two steps (figure 2), namely 1) the formation of [^{18}F]fluoroindole and 2) the reversed tryptophanase reaction[15] giving [^{18}F]-5-FTP. For the first step, 5-trimethylstannylindole will be investigated as a starting compound for fluorodestannylation to from 5-fluoroindole[16]. Fluorodestannylation is a method to label aromatic ring structures with F_2 gas from stannylated compounds. To investigate if 5-fluoroindole is a good substrate for the tryptophanase reaction, different

parameters like pH and concentrations of reactants will be used to optimize this latter reaction to obtain 5-FTP.

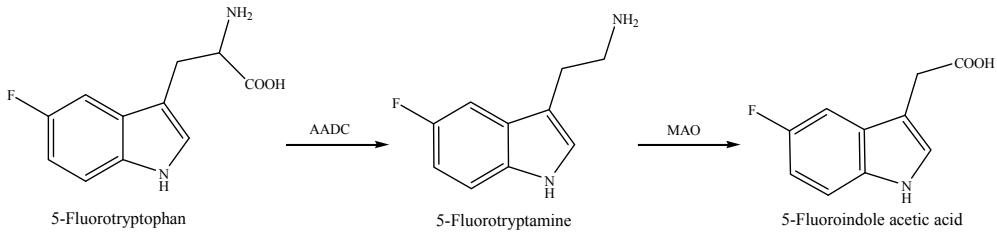

Figure 1. Tentative view on the metabolism of 5-fluorotryptophan.

The aim of these experiments is to investigate if an enzymatic synthesis of an ^{18}F tryptophan analogue is feasible, and if the resulting 5-fluorotryptophan is accumulated in a BON cell line.

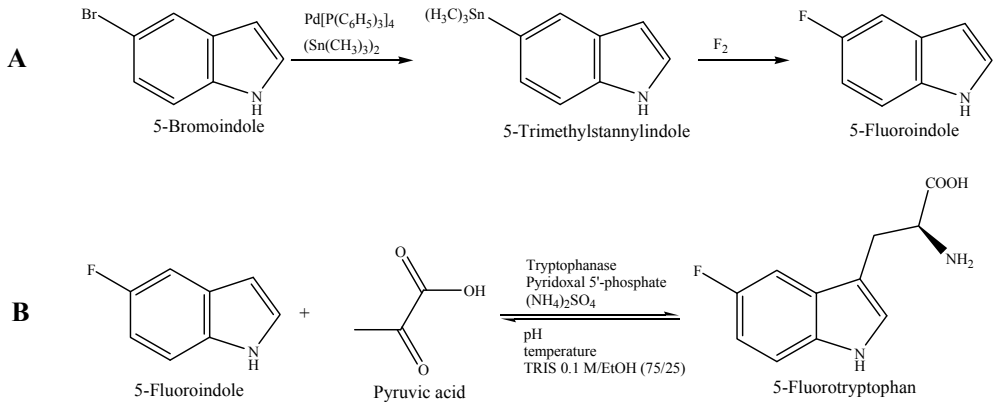

Figure 2. 5-Fluorotryptophan synthesis: A – Stannylation of 5-bromoindole and subsequent fluorination with F_2 gas; B – the reversed tryptophanase reaction applied on 5-fluoroindole.

Materials & Methods

Chemicals

Chemicals and solvents were obtained from Sigma (Zwijndrecht, The Netherlands), Merck (Amsterdam, The Netherlands), Janssen (Geel, Belgium) and Rathburn (Walkerburn, Scotland). 0.3 % F_2 in neon (6.2 bar) was used. Tris(hydroxymethyl)-aminomethane (TRIS) and ammonium sulfate (AMS) were dissolved in distilled water. Pyridoxal 5'-phosphate (PLP) was dissolved in 0.1 M sterile phosphate buffer pH 7.2. Tryptophanase (TRP) dissolved in 20 mM potassium phosphate buffer pH 7.5, 0.1 mM pyridoxal 5'-phosphate, 0.1 mM dithiotheitol and 20% glycerol was purchased from Ikeda (Hiroshima, Japan) and used without further treatment. GMC (5.6 mM *D*-glucose, 0.49 mM $MgCl_2$, 0.68 mM

$CaCl_2$) was added to phosphate-buffered saline solution (PBS; 140 mM NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4x2H_2O$, 0.2 mM KH_2PO_4). pH of the resulting PBS-GMC solutions was adjusted to 7.4 with sodium hydroxide. PBS-GMC was used to deplete internal amino acid pools[17,18].

Cell culture method

Cellular accumulation experiments were performed with cells derived from a human neuroendocrine pancreatic tumor (BON)[19]. Cells were maintained in 25 cm^2 culture flasks in 5 ml D-MEM/F-12 (1:1) medium supplemented with 10 % fetal calf serum (FCS). They were grown in a humidified atmosphere containing 5 % CO_2 and were routinely subcultured every 3-4 days. For experiments, the cells were grown to a concentration of 1.4 x 10^6 cells/ml. Viable cell number was determined by the trypan blue exclusion technique. Cell viability during and 1 hour after experiments was over 90%.

Analytical methods

Semipreparative HPLC purification:
System A: Column: Chromspher Si (250 x 10 mm), 5 µm; Eluent: Hexane/EtOAc : 9/1; Flow rate: 5 ml/min; Temperature: RT; UV detection at λ: 280 nm; Retention times: 5-trimethylstannylindole 13 min, 5-fluoroindole 14 min.
System B: Column: Econosphere C18 (250 x 10 mm), 10 µm; Eluent: NaH_2PO_4/EtOH: 9/1; Flow rate: 5 ml/min; Temperature: RT; UV detection at λ: 280 nm; Retention time 5-fluorotryptophan 19 min.
An electrospray mass spectrum was recorded on an API 3000 triple quadrupole mass spectrometer (MDS-Science, Concord, Ontario, Canada).
Nuclear magnetic response spectroscopy was performed on a Bruker 200 MHz .

Experimental

Cell experiments

Experiments were performed in 25 cm^2 culture flasks. Culture medium was removed and cells were washed with warm PBS (3 x 2 ml). PBS-GMC buffer (5 ml) containing the AADC inhibitor carbidopa (0.08 mM), the MAO A inhibitor clorgyline (0.1 mM) or the MAO B inhibitor pargyline (0.1 mM) was added. The cells were then placed in a water bath at 37°C. After 1 hour depletion period PBS-GMC buffer was removed and PBS-GMC buffer containing unlabeled 5-HTP or 5-FTP (55 µM) was added.
After 15 and 60 min of tracer incubation PBS-GMC buffer was removed. BON cells were harvested with 1 ml trypsin and taken up in 1 ml PBS containing 10% FCS. BON cells were washed three times with ice-cold PBS (1 ml) and centrifuged 10 min at 10,000 g. Lysis was performed with liquid nitrogen. Concentrations of 5-HTP and 5-FTP from lysed BON cells dissolved in 1 ml saline were determined as described earlier[20]. Results represent the mean of 3 experiments ± standard error of the mean (SEM).

Statistics

Differences between various groups (control and treated) were tested for statistical significance using Student's paired t-test. P-values < 0.05 were considered as significant.

Chemistry

*5-Trimethylstannyl-1H-indole (**1**)*

1.53 g 5-Bromoindole (7.8 mmol) was dissolved in 20 ml anhydrous 1,4-dioxane and 909 mg *tetrakis* triphenylphosphine palladium (0) (0.4 mmol) in 5 ml anhydrous 1,4-dioxane was added. After addition of 2.1 g hexamethylditin (15.3 mmol) the solution was refluxed for 2 days under argon. After cooling down, the mixture was taken up in 80 ml ethyl acetate, filtered and washed with water and brine. The organic residues were collected, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by silica gel chromatography (eluent: Hexane/EtOAc : 3/1; Rf: 0.79) to give 330 mg 5-trimethylstannyl-1*H*-indole as a solid in a yield of 15 %. Mp.: 48.3 °C. ^{1}H-NMR (CD_3OD, 298 K): 7.39 (s, 1H, H-4), 7.13 (m, 1H, H-7), 7.09 (m, 1H, H-2), 6.88 (m, 1H, H-6) 6.14 (m, 1H, H-3), 0.13 (s, 9H, H-8); ^{13}C NMR (CD_3OD, 298 K): 135.3 (C-8), 135.2 (C-5), 128.0 (C-4), 127.8 (C-3), 127.6 (C-6), 123.9 (C-1), 110.9 (C-7), 100.7 (C-2), -10.9 (C-9).

*5-Fluoroindole (**2**)*

20-35 mg (71-125 µmol) **1** was dissolved in 2 ml dry acetonitrile (from 4Å molecular sieves) and cooled down to 0 °C. 0.3 % F_2 in neon gas was bubbled through the solution at a flow rate of 200 ml/min within 10 min including one purge of pure neon gas. After heating at 50 °C for 10 min in a closed reaction vessel the mixture was filtered via a 22 µm LG pore filter. The mixture was purified by HPLC (system A) and evaporated to dryness with a rotary evaporator to obtain 5-fluoroindole.

Enzymatic synthesis of 5-fluorotryptophan

The reversed tryptophanase reaction is controlled by different parameters like the concentrations of used chemicals and enzyme, pH and reaction temperature (figure 2). Fixed parameters were the amount of 1.35 mg 5-fluoroindole (10 µmol), 50 µl TRP, 10 µl PLP 10.7 mM and 1.5 ml TRIS 0.1 M solvent containing 25 % ethanol. First, AMS 1.5 M was added to the solution of 5-fluoroindole followed by pyruvic acid. pH was then adjusted, tryptophanase and PLP were added and the mixture was incubated for 10 min. Reaction was stopped by addition of 2 drops HCl 6 M followed by filtration through a 22 µm GP pore filter and subsequent adjustment to pH ~ 9 and injection on HPLC system B. pH, temperature and the amounts of AMS 1.5 M and pyruvic acid were varied in several experiments to optimize yields. Yields were determined by HPLC. The experiment was repeated five times under optimal conditions times to determine yield as mean ± SEM.

*5-Fluorotryptophan (**3**)*

Purified **2** was dissolved in 1.5 ml TRIS 0.1 M/ethanol (75/25), 400 µl AMS 1.5 M and 20 µl pyruvic acid. The mixture of the pH was adjusted to pH 9.5-10.0 and 50 µl TRP and 10 µl PLP were added and warmed at 40 °C for 10 min in a metal heating block. The reaction was stopped by addition of 3 drops HCl 6 M and filtration on a 22 µm GP pore filter. The colorless clear mixture was purified on HPLC (system B) and the corresponding peak for 5-fluorotryptophan was analyzed by mass spectroscopy.

Results

Cellular accumulation of 5-HTP and 5-FTP

After 15 min of incubation, significant levels of 5-FTP (0.6 % of all 5-FTP in medium) were detected in cell lysates compared to 5-HTP (3.4 % of all 5-HTP in medium). After 60 min incubation period 5-FTP levels remained constant (0.5 % of all 5-FTP in medium) while 5-HTP levels increased over time (12.0 % of all 5-HTP in medium). Treatment with AADC and MAO inhibitors lowered 5-HTP levels after 60 min (carbidopa: 3.0 %, clorgyline: 1.5 %, pargyline: 3.1 % of all 5-HTP in medium) but led to an increase of 5-FTP levels (carbidopa: 0.9 %, clorgyline: 1.1 %, pargyline: 2.1 % of all 5-FTP in medium) (figure 3).

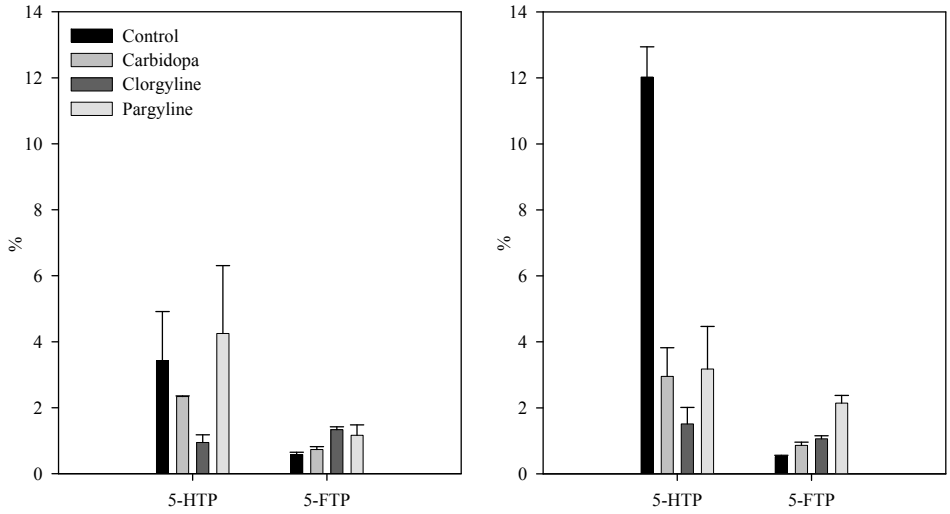

Figure 3. Accumulation of 5-HTP and 5-FTP in BON cells. Left: 15 min incubation time. Right: 60 min incubation time. Values express percentage of amounts 5-HTP and 5-FTP used for incubation. Mean ± SEM of 3 experiments.

Chemistry

5-trimethylstannylindole was obtained from its corresponding bromoindole in a yield of 15 %. ^{1}H-NMR analysis showed shifted signals for protons H2-H7 and a characteristic signal for the protons of the trimethylstannylgroup appearing in the product only. ^{13}C-NMR analysis showed a characteristic signal for the primary carbon atoms of the trimethylstannylgroup. Reaction of F_2 gas with 5-trimethylstannylindole gave 15 % yield of 5-fluoroindole. Optimal conditions for the synthesis of 5-FTP using the reversed tryptophanase reaction were determined by using 1.35 mg 5-fluoroindole dissolved in TRIS 0.1 M/ethanol (75/25) and addition of 400 µl AMS 1.5 M and 20 µl pyruvic acid. The effect on the pH on the 5-FTP yield was significant (figure 4). Maximal yields were found at 9.5-10.0. To estimate the yield, pH was adjusted to 9.5-10.0 and 50 µl TRP and 10 µl PLP were added and the whole mixture was incubated for 10 min at 40 °C. Under these conditions, yields of 73 ± 6 % were obtained after 5 experiments. The reversed

tryptophanase reaction was performed on 5-fluoroindole giving 5-FTP confirmed by mass spectroscopy of a HPLC sample resulting in a mass of 223.1 (mass 5-FTP +1: 223.1).

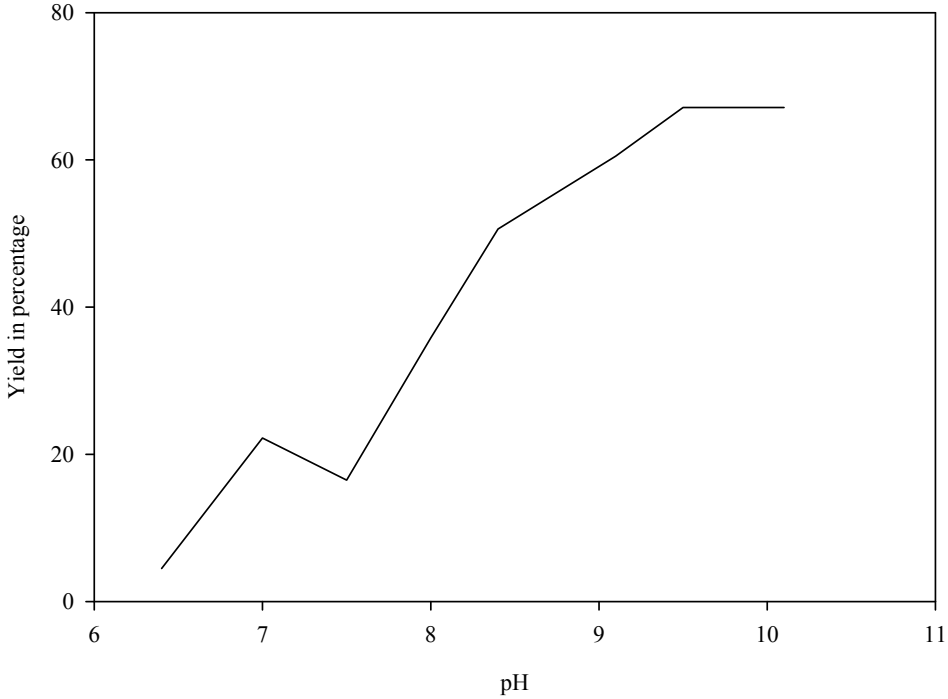

Figure 4. Enzymatic synthesis of 5-fluorotryptophan depending on pH. Optimal conditions were determined: 1.35 mg 5-fluoroindole in 1.5 ml TRIS 0.1 M/ethanol (75/25), 400 µl AMS 1.5 M, 20 µl pyruvic acid, 50 µl TRP, 10 µl PLP 10.7 mM, pH 9.5-10.0, 40 °C and 10 min reaction time. Yield after 5 experiments: 73 ± 6 %.

Discussion

The aim of this study was to develop an approach for the synthesis of the tracer [^{18}F]-5-FTP for the detection of neuroendocrine tumors with PET and to determine if 5-FTP accumulates in a BON cell line according to the APUD principle.

We showed that 5-FTP is taken up into the neuroendocrine pancreatic tumor cell line BON while cellular levels of 5-FTP were lower than 5-HTP levels. It would have been even more informative if in addition 5-fluorotryptamine levels, the cellular metabolic intermediate-product, were measured (figure 1). However as currently no suitable method is available, we were not able to identify 5-fluorotryptamine and 5-fluoroindole acetic acid in cell lysates. A reason for this could be the fact that fluorination of indoles probably leads to different retention times with our HPLC analysis.

Detection of 5-FTP proved to be possible in this setting, as shown. Quantification of the compounds 5-fluorotryptamine and 5-fluoroindole acetic acid is necessary to obtain more insight in the metabolic fate and cellular handling of 5-FTP. It can be envisaged that 5-FTP

is a substrate for AADC and that 5-fluorotryptamine is analogous to serotonin, stored in secretory granules[21,22].
The exposure of BON cells to specific AADC and MAO inhibitors led to an increase of cellular 5-FTP levels. From these data it can be concluded that uptake can be manipulated, but reasons for this increase could be further analyzed when also other metabolically related compounds are quantified in future experiments. A profiling method for the detection of 5-fluorotryptamine and other metabolites of 5-FTP can give more insights in intracellular accumulation.
A potential synthesis route to obtain 5-FTP is an enzymatic synthesis analogous to [^{11}C]-5-HTP. Tryptophanase activity is dependent on pH and temperature. Because of its fluorine atom, 5-fluoroindole has different physiological and charge properties than indole or 5-hydroxyindole. Therefore we determined if 5-fluoroindole was a good substrate for tryptophanase. High yields in the reversed tryptophanase reaction on 5-fluoroindole indicate that the fluorine atom was not detrimental to the enzymatic synthesis of 5-FTP from 5-fluoroindole. pH is the key factor in the enzymatic synthesis and if adjusted precisely, 5-FTP can be obtained in high and reliable yields.
As a prerequisite for this approach we investigated the synthesis of 5-fluoroindole by electrophilic fluorodestannylation. Major challenge is the separation of fluoroindole from its stannylated precursor, which is used in amounts of 20-35 mg. Simple solid phase extraction of precursor and product was not satisfying as both precursor and 5-fluoroindole were not sufficiently trapped on the extraction column. HPLC proved to be satisfactory. However, as described above, high amounts of precursor are used and therefore only small volumes of the reaction mixture have been injected on HPLC to avoid column capacity overload. We performed the enzymatic tryptophanase reaction with HPLC purified 5-fluoroindole giving 5-FTP, confirmed by mass spectroscopy. ^{18}F-labeling work towards [^{18}F]-5-FTP applying a similar approach is in progress.

Conclusion

5-FTP is accumulated in a NET cell line. 5-Fluoroindole is a good substrate for the enzymatic synthesis towards 5-FTP. 5-Trimethylstannylindole has been developed as useful precursor for the enzymatic synthesis towards ^{18}F labeled 5-FTP via 5-fluoroindole.

Acknowledgement

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