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Abstract

Reliable and non-invasive diagnostic tools are highly valuable for successful therapeutic strategies for the treatment of Alzheimer' s disease (AD). The existence of neurofibrillary tangles (NFTs) consisting of tau protein are one kind of the pathological features of AD, and its level of severity is correlated with the stage of AD. However, no clinically approved positron emission tomography (PET) probe is currently available for selective imaging of neurofibrillary tangles on patients. In this paper, we report our studies on biological characteristics of [18F]-THK523 as a novel tau imaging probe. With low molecular weight, [18F]-THK523 is stable, electrically neutral, lipophilic and non-mass concentration-dependent. Preliminary biological studies have shown the excellent properties of [18F]-THK523 as brain imaging tracer for further research.

Full Text

Preamble

Biological Characteristics of [18F]-THK523 for Tau Imaging

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Reliable and non-invasive diagnostic tools are highly valuable for successful therapeutic strategies for the treatment of Alzheimer' s disease (AD). Neurofibrillary tangles (NFTs) consisting of tau protein represent one of the key pathological features of AD, and their severity correlates with disease stage. However, no clinically approved positron emission tomography (PET) probe is currently available for selective imaging of neurofibrillary tangles in patients. In this paper, we report our studies on the biological characteristics of [18F]-THK523 as a novel tau imaging probe. With low molecular weight, [18F]-THK523 is stable, electrically neutral, lipophilic, and non-mass concentration-dependent. Preliminary biological studies have demonstrated excellent properties of [18F]-THK523 as a brain imaging tracer for further research.

Keywords: [18F]-THK523, Neurofibrillary tangles (NFTs), Alzheimer's disease (AD), Tau-specific probe, Biological characteristics

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Introduction

The National Institute on Aging-Alzheimer' s Association (USA) has suggested that Alzheimer' s disease would be optimally treated before significant cognitive impairment, defined as a "presymptomatic" or "preclinical" stage [**?**]. Diagnosis and treatment strategies for AD rely on sensitive and specific detection of incipient neuropathological characteristics, combined with emerging treatments that counteract molecular processes in AD pathogenesis. The hyperphosphorylation of tau protein and formation of intraneuronal neurofibrillary tangles represent characteristic neuropathological features in the AD brain.

Tau is a microtubule-associated protein (MAP) that localizes in neuronal axons and maintains microtubule stability, neurite outgrowth, and chromosome stability \cite{2-5}. NFTs consist of aggregated paired helical filaments (PHF) comprising aberrantly phosphorylated tau. NFTs first form in the entorhinal cortex at early AD stages and spread to the dentate gyrus, hippocampus, and cingulate cortex as memory loss develops [**?**]. NFTs, especially soluble hyperphosphorylated tau aggregates, interact with Aβ-mediated toxicity, oxidative stress, inflammation, and abnormal mitochondrial function [**?**, **?**]. Additionally, anti-tau treatment can reduce Aβ formation and excitotoxicity levels [**?**, **?**].

The utility of positron emission tomography, with a radioligand for translocator protein as a biomarker for tau-triggered toxicity, for tau imaging and diagnostic assessment of tauopathies with and without Aβ pathologies, is technically important for both clinical and basic research aimed at prodromal AD pathologies. Currently, no effective treatment targeting tau pathology is used clinically. Nakamura et al. [**?**] reported in 2012 that unlike trans p-tau, cis isomerization of p-tau by proline-directed kinases appears early in the brains

of humans with mild cognitive impairment, accumulates exclusively in degenerated neurons, and localizes to dystrophic neuritis during AD progression. The cis isomer cannot promote microtubule assembly, is more resistant to dephosphorylation and degradation, and is more prone to aggregation. Conventional peptidyl-prolyl cis-trans isomerases (PPIases) such as Pin1 can convert cis to trans p-tau to prevent Alzheimer' s tau pathology. A tau-specific PET probe can effectively evaluate such novel approaches through accurate, reliable, and reproducible noninvasive monitoring of tau protein aggregates in the living brain.

Increasing focus has been placed on developing PET imaging radiotracers for preclinical diagnosis of AD, particularly [18F]-THK523, which may be a potential tau-targeted probe. In vitro binding studies demonstrated that [18F]-THK523 had higher affinity to a greater number of binding sites on recombinant tau (k18$ $280k) than β-amyloid1−42 fibrils. [18F]-THK523 bound to tau pathology in autoradiographic and histofluorescence analysis of AD hippocampal serial sections. It showed higher retention in tau transgenic mouse brain than in wild-type littermates and bound to recombinant tau with much higher affinity than to β-amyloid plaques [?]. Furthermore, it demonstrated higher affinity to tau fibrils than Aβ fibrils when comparing the binding properties of [18F]-THK523 and other amyloid imaging agents, including PiB, BF-227, and FDDNP, to synthetic protein fibrils and human brain tissue [?]. Zeng et al. [?] demonstrated that [3H]-THK523 binds to NFTs and Aβ plaques in human AD brain sections; however, in transgenic mouse brain sections, [3H]-THK523 binds only to Aβ but fails to bind to NFTs. Okamura et al. [?] reported that novel 18F-labeled arylquinoline derivatives, 18F-THK-5105 and 18F-THK-5117, had higher binding affinity for tau protein aggregates and tau-rich AD brain homogenates, and higher brain uptake and faster clearance in normal mice than [18F]-THK523. In this paper, we report our complementary biological characteristics studies to investigate whether [18F]-THK523 can meet ligand criteria for tau imaging tracer.

Materials and Methods

A. Labeling Procedure

The protected precursor, 2-((2-(4-(tert-butoxycarbonyl)amino)phenyl)quinolin-6-yl)oxy)ethyl 4-methylbenzenesulfonate (THK-7), was synthesized in our lab [?]. [18F]-THK523 was radiosynthesized with high yield from THK-7 using a fully automated module (PET Science & Technology Co. Ltd., Beijing, China) [?]. Aqueous $18F^-$ trapped on a quadrupole mass analyzer (QMA) cartridge was washed with 1.5 mL of K_2CO_3 (2.73 mg/mL)/Kryptofix™2.2.2 (11.82 mg/mL), and the solvents were evaporated. After adding 2 mg of THK dissolved in 1 mL of acetonitrile (2 mg/mL), the nucleophilic substitution reaction was carried out at 120 °C. To hydrolyze the Boc protecting group, 250 µL of 1 N HCl solution was added. The mixture reacted at 105 °C for 5 minutes. Excess HCl was neutralized with 125 µL of 2 N NaOH. Saturated 1 N $NaHCO_3$ (125 µL) was added to adjust the pH to 7.4. The product was loaded onto a Sep-

Pak tC18 SPE cartridge and washed with water to remove free $18F^{-}$, polar byproducts, Kryptofix™2.2.2, etc. The cartridge was then washed with 2 mL of ethanol. Crude product was collected after passing through a sterile filter, followed by further purification using semipreparative high-performance liquid chromatography (Waters XBridge™ prep Shield RP18 10 µm, 250 mm × 10 mm, part No. 186003990, serial No. 101/123041GG01, 70% EtOH:30% H_2O; Waters Corporation, Milford, Massachusetts, USA) equipped with a Bioscan radioactivity detector at a flow rate of 4 mL/min and stabilized with ascorbic acid (2 mg, 0.011 mmol) before sterile filtration. Quality control of [18F]-THK523 was achieved by thin layer chromatography (TLC) and radio high-performance liquid chromatography (RHPLC).

Radiochemical yield of [18F]-THK523 was evaluated by TLC using silica gel G60 with fluorescence (F254) plates (cut into 10 cm × 0.4 cm strips) as stationary phase and ethyl acetate:n-hexane:triethylamine = 4:1:0.005 (V/V/V) as mobile phase. The reaction product was spotted with a capillary and developed by mobile phase. After development, the strips were dried at room temperature, cut into 1 cm × 0.4 cm pieces, and counted by a Wizard 1470 automatic gamma counter (Perkin Elmer Company, USA) equipped with a multi-channel analyzer. Retention factor (Rf) and labeling yield were determined from TLC chromatogram data. Two TLCs were run for each tested reaction condition and the data were averaged as the labeled rate.

The radiochemical purity (RCP) of [18F]-THK523 was determined by analytical RHPLC. The sample was passed through a Millipore filter carefully and then injected into the HPLC column (Purospher® STAR LPRP-18e endcapped (5 µm), 250$×$4.6 mm, sorbent Lot No. TA1752311, column No. 210072) at room temperature. The absorbance was measured at 350 nm and the flow rate was adjusted to 0.6 mL/min. An injection volume of 20 µL tracer was used with a mobile phase at a volume ratio of acetonitrile/water (containing 0.05% triethylamine) of 80%/20%. Retention time (Rt) was measured and checked against the standard product.

B. Electrophoresis

The charge of [18F]-THK523 was determined by paper electrophoresis using potassium phosphate buffer solution:alcohol:distilled water, 1:1:1 (V/V/V) with pH 7.4 as electrolyte and Xinhua No. 1 paper strips as support. The sample was run at a constant voltage of 110 V for 2.5 h. The strip was scanned by gamma counter. For comparison, a sample of $18F^{-}$ was run under identical conditions.

C. Determination of Lipid-Water Partition Coefficient of [18F]-THK523

The lipid-water partition coefficient of [18F]-THK523 was measured in two steps. Step 1: 1 mL of phosphate-buffered saline (PBS) (pH = 7.4) saturated with n-octanol and 1 mL of n-octanol saturated with PBS (pH = 7.4) were added to a

centrifuge tube containing 100 µL of sample. Step 2: The tube was capped and vortexed for 10 min at room temperature, then allowed to stand for 5 min. After reaching equilibrium, the tube was centrifuged at 2000 r/min (r = 6.0 cm) for 10 min. One hundred microliters of the organic phase and aqueous phase were pipetted out respectively and each phase was counted by the gamma counter. The organic phase of 500 µL was pipetted into another centrifuge tube, followed by addition of 500 µL of n-octanol saturated with PBS (pH = 7.4) and 1 mL of PBS (pH = 7.4) saturated with n-octanol. Step 2 was repeated six times. The partition coefficient was calculated as (cpm in organic phase)/(cpm in water phase).

D. Measurement of Plasma Protein Binding Rate

Heparin-anticoagulated fresh blood plasma from 10 volunteers was provided by the Nuclear Medicine Department, Huashan Hospital affiliated to Fudan University. Trichloroacetic acid with volume fractions of 10% and 25% was prepared. The experiment was divided into high, middle, mid-low, and low dose groups, each with four parallel samples. Each tube contained 0.2 mL blood plasma and 0.1 mL of [18F]-THK523 with activities of 22.20, 2.22, 0.22, or 0.02 MBq for the high, middle, mid-low, and low dose groups, respectively. After incubating for 2 hours at 37 °C, 1 mL of 25% trichloroacetic acid was added to each tube. The samples were vortex-blended and centrifuged at 2000 r/min (r = 6.0 cm) for 10 min. The supernatant was collected, then 1 mL of 10% trichloroacetic acid was added to the precipitate. This step was repeated twice. Plasma protein binding rate was calculated as: plasma protein binding rate = [(precipitation radioactive counts)/(precipitation + supernatant radioactive counts)] × 100%.

E. Stability Studies

Stability assessment of the complex was carried out by measuring its radiochemical purity at 25 °C. The radiochemical purity of [18F]-THK523 was determined by TLC and the radioactivity was counted by gamma counter at 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 7 h after preparation.

F. Blood Kinetic Studies

Blood clearance studies were performed in C57 mice (n = 5, (21 ± 1) g). For each animal, 5.18 MBq/140 µCi of [18F]-THK523 (0.1 mL) was administered intravenously through the tail vein. Blood samples (10 µL) were collected from the tail vein and radioactivity was measured by gamma counter at different time intervals (2, 5, 10, 15, 20, 30, 45, and 60 min) after injection. Data were expressed as percentage of administered dose at each time point. The weight of each blood sample was determined by weighing the microcentrifuge tube before and after collection. Radioactivity concentrations in blood were calculated as %ID/g. Blood clearance patterns of [18F]-THK523 were simulated using Pharmacokinetics Local Model (PLM) software developed by Cao et al. [**?**].

G. Micro PET Imaging

Normal C57 mice ((20 ± 2) g) were imaged with a Siemens Inveon PET/CT system (Siemens Medical Solutions, Knoxville, USA). After anesthesia induction and catheter placement, animals were positioned with their bodies in the center of the field of view and fixed in the scanner in prone, feet-first position (FFP). At the beginning of the PET scanning procedure, a CT scan (Inveon) was performed for all animals. [18F]-THK523 was administered via the catheter system intravenously in a slow bolus. The total applied volume was (0.18 ± 0.02) mL. The injected activity was (0.15 ± 0.03) mCi. Radioactivity in the syringe and catheter was measured immediately before and after injection. Dynamic data acquisition was performed using Inveon Acquisition Workplace (IAW, Siemens) for 60 min starting immediately after tracer injection (p.i.). PET images were reconstructed from 600 million coincidental 511 keV photon counts. Sinogram reconstruction yielded a 3D mapping of positron signal using Fourier rebinning and a 2D filtered back-projection algorithm with a ramp filter. Voxel size was set as 0.80 mm × 0.86 mm × 0.86 mm. CT images were reconstructed using a modified Feldkamp cone beam reconstruction algorithm (COBRA) from 360 projections with isotropic pixel size of 110 µm. Emission data were normalized and corrected for decay and dead time. Resulting sinograms were reconstructed with filtered back-projection (FBP) into 8 frames (1@120; 1@180; 3@300; 1@600; 2@900 s) for motion correction, ratio measurements, and image production for time-activity curve (TAC) generation.

For each micro PET scan, three-dimensional regions-of-interest (ROIs) were drawn over major organs using vendor software (Inveon Research Workshop; IRW) on decay-corrected whole-body images. All PET and CT image datasets were scaled to calibrated kBq/cc and saved in float format. Plane orientation was confirmed to radiological human brain standard such that the Z-axis was perpendicular to horizontal sections. To retrieve reliable small-animal PET results, accurate and standardized co-registration of PET to CT is essential. A two-step matching process was used: initial automatic rigid matching followed by manual adjustment if necessary. High-resolution CT scan was used as the basis for VOI definition. To quantify dynamic data, TACs with high initial time resolution were used.

H. Biodistribution Studies in Mice

Ex vivo biodistribution studies were carried out to confirm that quantitative tracer uptake values based on non-invasive micro PET imaging truly represented actual tracer distribution in normal mice. Fifty C57 mice ((20 ± 2) g) from Shanghai Slac Laboratory Animal Co. Ltd. were used in animal experiments (25 female, 25 male). They were divided randomly into ten groups according to sacrifice time points. [18F]-THK523 (0.1 mL) at an activity of 5.18 MBq/140 µCi was injected into the tail vein of each mouse, and animals were sacrificed at 2, 5, 10, 15, 30, 45, 60, 120, 180, and 240 min after injection. Samples of major organs/tissues of interest, including liver, spleen, pancreas, stomach,

intestine, femur, muscle, gonad, lung, kidney, heart, brain, and blood, were collected and wet-weighed. Specific radioactivity of tissue samples was measured using a gamma counter. Percent dose per organ was calculated by comparing tissue counts to counts of a suitably diluted aliquot of the injected material. Radioactivity concentrations in blood were also calculated as %ID/g.

All experiments were carried out in compliance with national laws for animal experimentation and were approved by the local committee for animal research.

Results

A. Electrophoresis

The charge of the complex was confirmed by paper electrophoresis. Table 1 shows that 95.8% of [18F]-THK523 remained stationary under current, indicating it was electrically neutral, while the $18F^-$ species moved toward the anode, indicating anionic behavior.

B. Determination of Lipid–Water Partition Coefficient of [18F]-THK523

The lipophilicity of [18F]-THK523 was determined by lipid–water partition coefficient (log P). Results are listed in Table 2 (log P = 0.99 ± 0.06, n = 7), indicating [18F]-THK523 is lipophilic, consistent with references [**?**, **?**].

C. Measurement of Plasma Protein Binding Rate

Plasma protein binding rates were (8.13 ± 0.35)%, (8.68 ± 0.45)%, (7.86 ± 0.32)%, and (8.11 ± 0.53)% for the high, middle, mid-low, and low concentration groups, respectively. These values did not differ significantly, indicating that the protein binding rate of [18F]-THK523 is not mass concentration-dependent.

D. Stability Studies

Stability of the radiolabeled compound over time was investigated. Radiochemical purity of [18F]-THK523 remained stable at approximately 90% for up to 5 h. It decreased to 87.6% and 86% at 6 h and 7 h, respectively (Fig. 1 [Figure 1: see original paper]). Considering radioactive decomposition and decay of ^{18}F, it is preferable to use [18F]-THK523 within 5 h after preparation.

E. Blood Kinetic Studies

The following dual-exponential equation was adopted to model the pharmacokinetics of [18F]-THK523 in mice:

$$Y = 2.23e^{-0.014t} + 1.89e^{-0.0007t}$$

where Y is %ID/g in blood and t is time in minutes. Distribution and elimination phases were consistent with compartment modeling results. Pharmacokinetic parameters of [18F]-THK523 are listed in Table 3 .

F. Micro PET Imaging and Biodistribution Studies in Mice

Biodistribution of [18F]-THK523 was determined ex vivo in healthy mice at 2, 5, 10, 15, 30, 45, 60, 120, 180, and 240 min after intravenous injection. Uptakes were highest initially at 2 min in liver ((7.96 $\pm$ 0.97) %ID/g), kidney ((4.32 $\pm$ 0.33) %ID/g), and heart ((4.18 $\pm$ 0.28) %ID/g), followed by rapid clearance (Fig. 2 Figure 2: see original paper). Preclinical study showed the highest [18F]-THK523 uptake in gall bladder, followed by liver, kidney, heart, and intestine, whereas femur and gonads showed the lowest uptake (Fig. 2(b)). Within less than 15 min, [18F]-THK523 essentially cleared from blood and plasma. [18F]-THK523 was mainly metabolized by the liver and excreted through the biliary system, leading to substantial rise in intestinal uptake at 15 min followed by slow decline starting at approximately 120 min. Micro PET imaging demonstrated these changes vividly in vivo, agreeing with ex vivo biodistribution analysis (Figs. 2(a) and 2(c)). Bone uptake rose then decreased slowly due to radioactive decomposition, which could be improved with ascorbic acid (2 mg, 0.011 mmol).

Brain uptake was (2.62 $\pm$ 0.39) %ID/g at 2 min after injection. Mouse brains were dissected into the following regions: cortex (frontal cortex, parietal cortex, temporal cortex, occipital cortex), striatum, hippocampus, thalamus, cerebellum, pons, and medulla oblongata. Uptakes were highest initially at 2 min in occipital cortex ((4.91 $\pm$ 0.94) %ID/g), temporal cortex ((3.33 $\pm$ 0.72) %ID/g), and hippocampus ((3.07 $\pm$ 0.35) %ID/g), followed by rapid clearance (Figs. 3(a) and 3(b)). Tracer uptake in occipital and temporal cortices was higher than in other cortical regions (Fig. 3(b)). The brain uptake trend of [18F]-THK523 was similar between ex vivo and in vivo measurements from injection to 60 min. The highest brain uptake occurred at 2 min after injection, followed by quick clearance during the first 90 min post-injection. Clearance was relatively slow from 90 min to 240 min after injection (Fig. 4 [Figure 4: see original paper]).

Discussion

NFTs are pathological hallmarks found in AD brains and are closely associated with dementia severity, indicating their contribution to neuronal dysfunction. As therapeutic targets for disease-modifying therapy in AD, PET tracers for imaging NFTs in the brain would be valuable for developing new AD therapies.

Considering factors related to brain uptake, a radioactive tracer can hardly cross the blood-brain barrier (BBB) if it is not electrically neutral or lipophilic. For brain tracers, low plasma protein binding rate is also critical for brain uptake. If plasma protein binding is too high, the tracer cannot access target regions. The analytical data and favorable log P suggest that [18F]-THK523 should cross the BBB and enter the central nervous system. Additionally, with

ascorbic acid present, [18F]-THK523 is stable at room temperature for up to 5 h. These biological characteristics demonstrate that [18F]-THK523 satisfies basic requirements for a brain tracer. Blood kinetic studies show that [18F]-THK523 distributes quickly from blood to other organs ($t_{1/2\alpha} = 47.9$ min) with relatively favorable retention time in target organs ($t_{1/2\beta} = 965.1$ min).

Metabolism and biodistribution patterns should be evaluated for any radiopharmaceutical candidate considered for clinical translation. Our ex vivo studies reveal high brain uptake at 2 min after injection, especially in the hippocampus, followed by rapid clearance in healthy mice. In vitro and in vivo studies have confirmed that [18F]-THK523 has high affinity and selectivity for tau pathology [**?**, **?**], and our biological characteristics results should help fulfill its brain ligand criteria for further imaging trials.

Conclusion

[18F]-THK523 was radiosynthesized on an automated module and its biological characteristics were evaluated. In vitro studies demonstrated that [18F]-THK523 was electrically neutral, lipophilic (log P = 0.99 ± 0.06, n = 7), and quite stable, maintaining radiochemical purity above 90% for up to 7 h at room temperature. Due to its low molecular weight, [18F]-THK523 can easily cross the blood-brain barrier. With relatively low plasma protein binding rate, [18F]-THK523 is not mass concentration-dependent. Pharmacokinetic parameters from blood kinetic studies yielded the dual-exponential equation $Y = 2.23e^{-0.014t} + 1.89e^{-0.0007t}$ with $t_{1/2\alpha} = 47.9$ min, $t_{1/2\beta} = 965.1$ min, $K_{12} = 0.0067$ min^{-1}, $K_{21} = 0.0070$ min^{-1}, $K_e = 0.0015$ min^{-1}, plasma clearance = 0.036 %ID/g/min, and area under concentration-time curve = 2785.1 ID%/g/min. [18F]-THK523 shows high brain uptake, and our biodistribution study in healthy C57 mice demonstrates that it is mainly metabolized by the liver and excreted through the biliary system. [18F]-THK523 may be a promising candidate for molecular imaging of tau pathology.

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