[18F]Fluoroazabenzoxazoles as potential amyloid plaque PET tracers: synthesis and in vivo evaluation in rhesus monkey


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Abstract

Introduction: An 18F-labeled positron emission tomography (PET) tracer for amyloid plaque is desirable for early diagnosis of Alzheimer’s disease, particularly to enable preventative treatment once effective therapeutics are available. Similarly, such a tracer would be useful as a biomarker for enrollment of patients in clinical trials for evaluation of antiamyloid therapeutics. Furthermore, changes in the level of plaque burden as quantified by an amyloid plaque PET tracer may provide valuable insights into the effectiveness of amyloid-targeted therapeutics. This work describes our approach to evaluate and select a candidate PET tracer for in vivo quantification of human amyloid plaque.

Methods: Ligands were evaluated for their in vitro binding to human amyloid plaques, lipophilicity and predicted blood–brain barrier permeability. Candidates with favorable in vitro properties were radiolabeled with 18F and evaluated in vivo. Baseline PET scans in rhesus monkey were conducted to evaluate the regional distribution and kinetics of each tracer using tracer kinetic modeling methods. High binding potential in cerebral white matter and cortical grey matter was considered an unfavorable feature of the candidate tracers.

Results: [18F]MK-3328 showed the most favorable combination of low in vivo binding potential in white matter and cortical grey matter in rhesus monkeys, low lipophilicity (Log D=2.91) and high affinity for human amyloid plaques (IC50=10.5±1.3 nM).

Conclusions: [18F]MK-3328 was identified as a promising PET tracer for in vivo quantification of amyloid plaques, and further evaluation in humans is warranted.

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Keywords: Alzheimer’s disease; Amyloid plaque, PET; Monkey; Fluorine-18; White matter

1. Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disorder, with about 27 million estimated worldwide cases of AD in 2006 [1]. The prevalence of AD is predicted to quadruple by 2050 due to an aging population, and new diagnostic paradigms are needed since AD diagnosis is often uncertain, making early and/or preventative treatment of AD a challenging task [2,3]. Moreover, current therapeutics for AD are palliative in nature and only provide a temporary slowing of inevitable cognitive decline [4]. One of the pathological hallmarks of AD is the parenchymal deposition of cerebral amyloid plaques, which are composed of aggregated fibrillar β-amyloid proteins in two primary isoforms: Aβ(1–40) and Aβ(1–42) [5]. Therefore, noninvasive methods of measuring β-amyloid aggregates may serve as a biomarker for AD and expedite development of novel therapies aimed at reducing β-amyloid production or increasing its clearance. For instance, inhibition of beta-secretase or gamma-secretase, the enzymes responsible for production of Aβ(1–40) and Aβ(1–42), is of high interest as a potential disease-modifying therapy [4]. A positron emission tomography (PET) tracer for in vivo quantification of amyloid plaques would enable more confident selection of the appropriate patients for clinical trials of potential amyloid plaque...
modifying therapies, especially for patients in the early stages of AD. Additionally, an amyloid plaque PET tracer may be useful for monitoring changes (or lack thereof) in amyloid plaque burden over time and correlating these changes to disease progression and therapeutic effect. Finally, if successful AD therapies are developed, such a PET tracer could be used to identify AD patients earlier in the course of disease, which could increase the probability of successful treatment.

Many potential amyloid plaque PET tracers have been evaluated preclinically, but only a few tracers have been evaluated clinically that have shown the ability to differentiate AD patients from healthy elderly subjects. Several recent reviews have effectively summarized these preclinical and clinical efforts [6–10]. The first and most widely studied of these clinically validated imaging agents is [11C]PIB [11]. However, for multicenter clinical trials, the short half-life ($t_{1/2} = 20$ min) of the $^{11}$C radiolabel of [11C]PIB is a limiting factor. The PET tracer must be produced and utilized on-site, near the cyclotron used for generation of the $^{11}$C radioisotope. Consequently, there has been great interest in discovering and developing an $^{18}$F-labeled amyloid plaque PET tracer. The longer half-life of $^{18}$F ($t_{1/2} = 110$ min) provides adequate time for production of the PET tracer at a central site and subsequent distribution of the radioligand to remote sites. This allows for broader access to AD patients that would be otherwise unavailable for clinical studies or AD therapeutics that require a diagnostic PET tracer. Efforts in developing an $^{18}$F-labeled amyloid plaque PET tracer have led to the discovery and development of $^{18}$F-AV-45, which is currently the most thoroughly characterized and clinically advanced $^{18}$F-labeled amyloid plaque PET tracer [12]. $^{18}$F-AV-45 has been shown to effectively differentiate AD patients from healthy control subjects, and the binding of $^{18}$F-AV-45 in AD patient brains has been correlated postmortem with amyloid plaque deposits [3, 13]. However, $^{18}$F-AV-45 and other amyloid plaque tracers display high white matter uptake that may be a liability in the identification of early-onset AD [3]. Herein we describe our application of PET studies in rhesus monkeys to aid in identification of $^{18}$F-MK-3328, an amyloid plaque PET ligand with low white matter uptake in rhesus monkey brain.

2. Materials and methods

2.1. Materials

$^{18}$F-Fluoride was purchased from Siemens Molecular Imaging Biomarker Research (North Wales, PA, USA) and transported to the radiochemistry laboratory on an anion exchange resin in a lead container. [3H]DMAB, [3H]MK-3328, [3H]AD-269 and [3H]PIB were prepared by Merck Research Laboratories (Rahway, NJ, USA). Solvents were of anhydrous grade and were purchased from Aldrich (Milwaukee, WI, USA) or Acros (Somerville, NJ, USA). The acetonitrile (MeCN) used for high-performance liquid chromatography (HPLC) was Optima grade from Fisher Scientific (Pittsburgh, PA, USA). Ketamine (Ketaset) was obtained from Fort Dodge (Fort Dodge, IA, USA), and propofol (Rapinovet) was obtained from Schering-Plough Animal Health (Union, NJ, USA). All other reagents were obtained from Aldrich. Precursors and unlabeled reference standards were prepared by Merck Research Laboratories (West Point, PA, USA) according to published methods [14, 15]. The microwave apparatus Model 521A was obtained from Research Instruments (Skokie, IL, USA). Radiochemical procedures were carried out remotely in a lead hot cell using a modified Gilson 233XL liquid handler [16]. The radiotracers were purified by reverse-phase HPLC using a Waters Model 600E controller (Milford, MA, USA). The preparative HPLC runs were monitored at 254 nm using a Pharmacia-Biotech (Piscataway, NJ, USA) UV-MII UV detector and a Bioscan (Mississauga, Ontario, Canada) FlowCount photodiode radiodetector. The radiochemical purity and identity of the tracers were determined by an independent coinjection with the authentic standard on an analytical HPLC system (Waters 600E) equipped with a Waters 996 UV detector (254 nm) and a FlowCount photodiode radiodetector (Bioscan). The frozen human brain samples of AD and non-AD subjects were purchased from Analytical Biological Services Inc. (Wilmington, DE, USA).

2.2. IC$_{50}$ determinations

Brain homogenates of frontal cortex from AD patient brain donors were prepared by homogenizing the frontal cortex in ice-cold phosphate-buffered saline (PBS), pH 7.4, using a Polytron at setting 5 for 30 s at 4°C. The final concentration of brain homogenates was 10 mg wet tissue per 1 ml buffer. Homogenates were aliquoted at 5 ml/tube and stored at $-70$°C prior to use. The specific activity of [3H]AD-325 was 82.3 Ci/mmol in a volume of 3.76 mCi/ml, and the final concentration of radioligand for tissue homogenate binding assay was 5.0 nM. Brain homogenates were diluted with PBS buffer to 0.2 mg/ml from the original 10-mg/ml volume, and 200 μl was used in the assay for a final concentration of 40 μg per assay tube. Unlabeled test compounds were dissolved in DMSO at 1 mM. Dilution of test compound to various concentrations from 1×10$^{-6}$ to 1×10$^{-11}$ M was made with PBS containing 2% DMSO. Total binding was defined in the absence of competing compound, and nondisplaceable binding was determined in the presence of 10 μM unlabeled self-block. Compound dilutions (10×) were added (25 μl per tube) containing 200 μl brain homogenate dilution, the tubes were preincubated at room temperature (RT) for 10 min, and then radioligand dilutions (10×) were added to the assay tubes (25 μl per tube) for a final volume of 250 μl per tube. Incubation was carried out at RT (25°C) for 90 min, and the assay samples were filtered onto GF/C filters using a Skatron 12 well harvester, washing
on setting 5–5–5 (3×2 ml) in ice-cold buffer (PBS, pH 7.4). GF/C filter papers were presoaked in 0.1% bovine serum albumin (BSA) for 1 h at RT before use. Filters were punched into scintillation vials and counted in 2 ml Ultima Gold on Perkin Elmer Tri-Carb 2900TR for 1 min. All assays were performed in triplicate, and the data were analyzed using Prism software.

2.3. Log D determinations

Log D values were determined using an HPLC-based method. Samples were analyzed using a reversed-phase column (Waters XTerra MS C18, 3.5 µm, 3×30 mm) and a mobile phase of MeCN (A) and phosphate buffer, pH 7.4 (B), with a flow rate of 1.5 ml/min. A gradient mobile phase of MeCN (A) and phosphate buffer, pH 7.4 (B), with a flow rate of 1.5 ml/min. A gradient mobile phase was used, which began at 95% B, maintained for 0.2 min and decreased linearly to 2% B over 1 min, and then maintained at 2% B for 0.4 min. Log D values were determined by comparing the retention time of the compound of interest to that of standards having known log D (octanol/H2O partition coefficient at pH=7.4) values. The standards used were uracil, atenolol, caffeine, warfarin, ketoconazole, tamoxifen, captopril, hydrocortisone, carbamazepine, imipramine, terfenadine and simvastatin dissolved in MeCN/methanol/DMSO (10/80/10). Using the retention time of the first peak (uracil) as t₀, the log k' was calculated for the remaining 11 compounds using their retention time (tR) and the following equation: log k'=log ((tR–t₀)/t₀). The best-fit curve was calculated for experimental log D vs. log k' using the experimental log D values. Compounds of interest were analyzed, and the derived log k' was used to determine the log D using the best-fit curve.

2.4. Passive cell permeability and P-glycoprotein transport ratio

Passive cell permeability was evaluated across monolayers of LLC-PK1 cells and was calculated by the following formula for samples taken at t=3 h:

\[ P_{app} = \frac{\text{volume of receptor chamber (ml)}}{\text{area of membrane (cm²)}} \times \frac{\Delta \text{in concentration} (\mu M)}{\Delta \text{t in time (s)}} \]

The P-glycoprotein (P-gp) transport ratio, i.e., the ratio of permeabilities across each direction of the cell monolayer, for all compounds was evaluated across monolayers of LLC-PK1 cells overexpressing human P-gp [17]. The P-gp transport ratio was determined at 3 h for each compound. Verapamil, a known P-gp substrate, was evaluated as a positive control.

2.5. Determination of radiochemical purity and specific activity

Positron emission tomography tracer radiochemical purity and specific activity were determined by injection on a Waters 600E HPLC system equipped with a Waters 996 UV detector (254 nM) and a photodiode radiodetector (Bioscan FlowCount). A Waters XTerra C18 column (4.6×150 mm, 5 µm) was used with MeCN (solvent A) and 95:5 MeCN:H2O (0.1% TFA) (solvent B) at 1 ml/min. A 15-min linear gradient of 20:80 A:B to 90:10 A:B was used. Positron emission tomography tracer radiochemical purity and specific activity were determined by injection on a Waters 600E HPLC system equipped with a Waters 996 UV detector (254 nM) and a photodiode radiodetector (Bioscan FlowCount). A Waters XTerra C18 column (4.6×150 mm, 5 µm) was used with MeCN (solvent A) and 95:5 MeCN:H2O (0.1% TFA) (solvent B) at 1 ml/min. A 15-min linear gradient of 20:80 A:B to 90:10 A:B was employed. Radiochemical purity was determined by calculating the percent of radioactivity attributed to the PET tracer in the radioactive HPLC trace after integration of all radioactive peaks. The specific activity of the PET tracer was determined as follows: an aliquot (~0.1 ml) of the tracer was counted for radioactivity, corrected for decay from end of synthesis and evaluated by analytical HPLC. The HPLC UV response was measured against a calibration curve that was prepared with the unlabeled reference standard to determine the mass associated with the decay-corrected radioactivity of the injected aliquot.

2.6. Preparation of [18F]fluoride eluting solution

To a solution of K2CO3 (3 mg) in deionized H2O (5 ml) was added potassium oxalate (200 mg), and the mixture was stirred until dissolved. A total of 0.5 ml of the resulting solution was diluted with deionized H2O (2.5 ml) and MeCN (12 ml) to provide a 15-ml stock of [18F]fluoride eluting solution.

2.7. Radiosynthesis of [18F]MK-3328

An anion exchange resin containing [18F]fluoride (typically, ~1 Ci) was eluted with 0.7 ml of [18F]fluoride eluting solution into a 1-ml v-vial in the microwave cavity. This vial was vented using an 18-gauge 1” syringe needle attached to a gas bag. To the aqueous [18F]fluoride solution was added Kryptofix 2.2.2 (0.2 ml, 36 mg/ml in MeCN), and the [18F]fluoride was dried under argon flow using microwave pulses (70°C, 35 W) to heat the aqueous MeCN. Additional aliquots of MeCN (2×0.5 ml) were added, and the azetotropic drying process was repeated. Chloropyridine I (2.0 mg, 7.0 µmol) was added to DMSO (0.25 ml) in an autosampler vial and gently heated until dissolved. The precursor solution was added to the microwave vial, and the reaction mixture was heated by the microwave for 4 min at 140°C, 60 W. After cooling to <40°C, the reaction mixture was diluted with MeCN:H2O (0.4 ml, 60:40) and purified by HPLC [Phenomenex Gemini C18 column (10×150 mm, 5 µm), MeCN (solvent A) and 10 mM Na2HPO4 (solvent B)] under isocratic conditions of 45:55 A:B at 5 ml/min. The peak corresponding to [18F]MK-3328 was collected (retention time of ~9 min), the MeCN was removed in vacuo, and the remainder was transferred to a capped vial using physiologic
saline as a rinse to give $[^{18}F]$MK-3328 with an average yield of 14%±13% (determined from integration of the radioactive HPLC trace), a specific activity of 2471±1389 Ci/μmol and a radiochemical purity of >98% (n=25).

2.8. Radiosynthesis of $[^{18}F]$AD-278

$[^{18}F]$AD-278 was prepared using the procedure for $[^{18}F]$MK-3328, substituting chloropyridine 2 (1.0 mg, 3.5 μmol) as the precursor. The semipreparative HPLC solvent system used was 50:50 MeCN:Na₂HPO₄ (0.01 N) at 5 ml/min, and the retention time was ~13 min. The product was isolated with a yield of 40%±18% (n=4), radiochemical purity of >99% and specific activity of 1032 Ci/μmol (n=2).

2.9. Radiosynthesis of $[^{18}F]$AD-269

$[^{18}F]$AD-269 was prepared using the procedure for $[^{18}F]$MK-3328, substituting chloropyridine 3 (1.0 mg, 3.7 μmol) as the precursor. The semipreparative HPLC solvent system used was 55:45 MeCN:Na₂HPO₄ (0.01 N) at 5 ml/min, and the retention time was ~9 min. The product was isolated with a yield of 35%±18% (n=4), radiochemical purity of >99% and specific activity of 5023±2927 Ci/μmol (n=4).

2.10. Radiosynthesis of $[^{18}F]$AD-265

$[^{18}F]$AD-265 was prepared following the procedure for $[^{18}F]$MK-3328 with the following modifications. Boc-protected chloropyridine 4 (1.0 mg, 2.8 μmol) was used as the precursor. After heating the precursor in the microwave for 4 min at 140°C (65 W), 1 M HCl (0.2 ml) was added. The reaction was heated at 80°C (35 W) further for 5 min followed by addition of 1 M NaOH (0.22 ml). The reaction mixture was diluted with MeCN: H₂O (0.4 ml, 60:40) and purified by HPLC [Phenomenex Gemini C18 column (10×150 mm, 5 μm), MeCN (solvent A) and 10 mM Na₂HPO₄ (solvent B)] under linear gradient conditions of 30:70 A:B to 70:30 A:B in 15 min at a flow rate of 5 ml/min. The desired product was collected at a retention time of ~12 min, $[^{18}F]$AD-265 was isolated with a yield of 10%±3% (n=3) and a radiochemical purity of >98% (n=2).

2.11. Radiosynthesis of $[^{18}F]$AV-45

$[^{18}F]$AV-45 was synthesized similar to methods previously reported [12]. Briefly, (E)-2-2-(2-(2-methanesulfonyloxyethoxy)ethoxy)ethoxy)-5-(4-methylaminostyryl)pyridine (1 mg, 2.3 μmol) was dissolved in DMSO (0.25 ml) and heated in a microwave cavity for 2.5 min at 100°C (50 W). Subsequently, 1 M HCl (0.2 ml) was added, and the mixture was heated at 80°C (35 W) further for 5 min followed by addition of 1 M NaOH (0.22 ml). The reaction mixture was purified by HPLC [Phenomenex Gemini C18 column (10×150 mm, 5 μm), MeCN (solvent A) and 10 mM Na₂HPO₄ (solvent B)] under isocratic conditions of 50:50 A: B at a flow rate of 5 ml/min. The desired product was collected at a retention time of ~10 min. $[^{18}F]$AV-45 was isolated with a yield of 39%, a specific activity of 994 Ci/μmol and a radiochemical purity of >98% (n=1).

2.12. Imaging protocol and animal care

All monkey PET imaging studies were conducted under the guiding principles of the American Physiological Society and the Guide for the Care and Use for Laboratory Animals published by the US National Institutes of Health (publication no. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at Merck Research Laboratories (West Point, PA, USA). Rhesus monkeys (R352: 13 kg, age 8 years; R460: 11 kg, age 7 years) were initially sedated with ketamine (10 mg/kg intramuscularly), induced with propofol (5 mg/kg intravenously), intubated and respired with medical grade air at ∼10 cc/breath/kg and 20 respirations per minute. The anesthesia was maintained with propofol (0.4 mg/kg/min) for the duration of the study. The PET scans were performed on an ECAT EXACT HR+ (CTI/Siemens, Knoxville, TN, USA) in three-dimensional mode. Transmission data for attenuation correction were acquired in two-dimensional mode before injection of the radiopharmaceutical. Emission scans were performed for 90 min following bolus intravenous injection of the PET tracer and consisted of 22 frames with a progressive increase in frame duration (4×15, 4×60, 5×180, 4×300 and 5×600 s). Baseline PET scans were acquired for $[^{18}F]$MK-3328, $[^{18}F]$AD-269 and $[^{18}F]$AD-278 in monkey R352 and monkey R460. Baseline PET scans were acquired for $[^{18}F]$AD-265 and $[^{18}F]$AV-45 in monkey R352 only. Animals were scanned on a Siemens Trio 3T magnet. T1-weighted MPRAGE pulse sequence (TR/TE/TI/FA=1.47/ 4.38/870/12) was used to obtain high-resolution images (0.5×0.5×0.8 mm³) of the animals’ whole heads for merging with summed PET images.

2.13. Image processing and evaluation

For each animal, PET images were obtained by integrating all frames in the dynamic acquisition under baseline conditions. Regions of interest (ROIs) were drawn in the cerebellar cortex (cerebellum), neocortex (cortex), thalamus/midbrain and white matter. The cortex and white matter regions were defined by cluster analysis, where differences in tracer kinetics between the white matter and grey matter were utilized to help define tracer uptake in the white matter vs. tracer uptake in the cortical grey matter. This was achieved by implementing cluster analysis on the dynamic PET data using the k-means clustering algorithm (MATLAB function ‘kmeans’). The algorithm places the TAC for each voxel in the brain in the cluster with the nearest mean. The number of clusters is identified by the user using a criterion such as the average sum of squared error (SSE) between cluster TACs and constituent voxel curves. The average SSE saturates after a certain number of clusters, following which visual inspection of the clusters is made to select the optimal number of clusters for the data
Using this method, the ROI for white matter was extended to the edge of the cortical grey matter ROI (Fig. 4). Similarly, the cerebellar cortex region was carefully defined using cluster analysis to avoid signal spillover from the nearby cerebellar nuclei.

Tissue time–activity curves (TACs) were obtained by projecting the defined ROI onto all frames of the dynamic PET scans and expressed in SUV by using the animal’s weight and the corresponding tracer injected dose: $TAC_{SUV}=1000 \times TAC \ (Bq/ml) \times \text{weight (kg)} / \text{tracer dose (Bq)}$.

Alignment between PET studies and TAC creation were performed using analysis software developed in-house and written in Matlab (The MathWorks, Inc., Natick, MA, USA).

### 2.14. Binding potential determination

Binding potentials (BPs) were calculated using Logan graphical analysis using the cerebellar cortex as a reference region [19].

### 2.15. In vitro autoradiography

Frozen brain slices (20-μm thickness) from human AD, normal control subjects (non-AD) or rhesus monkey (25 years old) were prepared using a cryostat (Leica CM3050) and kept in sequential order. The tissue slices were placed on Superfrost Plus glass slides (catalog no. 5075-FR, Brain Research Laboratories, USA), dried at RT and stored at −70°C before use. On the day of a binding experiment, adjacent slices were selected from each brain region of interest for in vitro autoradiographic study and were thawed at RT for 15 min. The brain slides were first preincubated at RT for 20 min in PBS buffer, pH 7.4. The slices were then transferred to fresh buffer containing radioligand (1.0–5.0 nM) or radioligand plus competitor (1.0 μM) as described above, and incubated at RT for 90 min. Incubation was terminated by washing the slices 3×3 min in ice-cold (4°C) buffer (PBS, pH 7.4). After washing, the slices were briefly rinsed in ice-cold (4°C) deionized H₂O and then dried completely by an air blower at RT. The slices were placed against Fuji Phosphor Image Plates (TR25, Fuji) in a sealed cassette for exposure at RT. After 1 week of exposure, the plates were scanned in Fuji BAS 5000 Scanner, and the scanned images were analyzed using MCID 7.0 software. [³H]-microscales (Amersham Biosciences, GE) were used for quantification of radioligand binding density. Total binding of radioligand in brain slices was defined in the absence of competitor (total binding), and nonspecific binding was determined in the presence of competitor.

### 2.16. Immunohistochemistry

To verify the presence or absence of amyloid plaques, all tissue samples used in these studies were sectioned and analyzed by immunohistochemistry using a mouse anti-human β-amyloid monoclonal antibody (clone 6E10; Convance, Princeton, NJ, USA). Briefly, 14-μm-thick cryosections were mounted onto Superfrost Plus slides (Fisher Scientific), fixed for 10 min in ice-cold acetone: ethanol (3:1) and washed in PBS. Sections were treated with 3.0% hydrogen peroxide for 20 min to inactivate endogenous peroxidases, washed in PBS and incubated with blocking buffer (5.0% goat serum containing 1.0% BSA, 0.1% fish skin gelatin, 0.1% Triton X-100, 0.1% Tween 20 and 0.05% NaN₃) for 1 h at RT to inhibit nonspecific antibody binding. After incubating at 4°C with or without the 6E10 antibody (0.08 μg/ml) for 18 h, the slides were washed thoroughly in PBS. Tissue-bound 6E10 antibody was detected with sequential incubations in peroxidase-conjugated goat anti-mouse IgG polymer (Envision+, Dako Corp., Carpinteria, CA, USA) and diaminobenzidine tetrahydrochloride chromogen. The slides were then counterstained with hematoxylin.

### 3. Results and discussion

Four fluoroazabenzoxazoles were synthesized and characterized in vitro for their suitability as amyloid plaque PET tracers (Fig. 1 and Table 1). All four compounds showed high binding affinity in human AD brain cortical homogenates vs. [³H]DMAB. AD-278 was the most potent ligand (IC₅₀=4 nM), AD-269 and MK-3328 had slightly lower potencies (IC₅₀=8 and 10.5 nM, respectively), and AD-265 was the least potent ligand (IC₅₀=17 nM). AD-278, AD-269 and MK-3328 showed no specific binding to

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Fig. 1. Structures of [³H]PIB, [¹⁸F]AV-45, [³H]DMAB and four amyloid plaque PET tracer candidates.
control cortical brain homogenates that were negative for amyloid plaques as determined by staining with the 6E10 antibody (data not shown). This indicates the specificity of these ligands for binding amyloid plaques. The lipophilicity was moderate for all four ligands, with log D measurements ranging between 2.94 and 3.52. Lipophilicity measurements in this range would be expected to provide good passive cell permeability, but not display prohibitory levels of nondisplaceable binding.

An amyloid plaque ligand must rapidly cross the blood–brain barrier (BBB) and therefore should have good passive cell permeability. Additionally, they should be poor substrates for P-gp, which is a key efflux pump at the BBB. A commonly used, well-validated assay for determining cell permeability and P-gp susceptibility is by measurement of the bidirectional transport of compounds across LLC-PK1 cell monolayers expressing high levels of human P-gp [17,20]. In this assay, we consider cell permeability rates <10×10⁻⁶ cm/s as low, while rates >20×10⁻⁶ cm/s are considered good and rates >40×10⁻⁶ cm/s are very high.

P-glycoprotein ratios of less than 3 are considered favorable, and ratios can range as high as 30 for strong P-gp substrates. None of the candidate amyloid plaque ligands were good substrates for human P-gp, and their cell permeability was high. As such, all would be expected to rapidly penetrate the BBB and were good candidates for in vivo evaluation in rhesus monkey.

Following the encouraging potency, lipophilicity and cell penetration data from the in vitro studies with the candidate ligands, methods for radiolabeling with [18F] were explored (Fig. 2). Although nitro is typically a more reactive leaving group for synthesis of 2-[18F]fluoropyridine derivatives [21], the analogous chloro compounds were utilized as precursors since they were more readily synthesized [14]. Additionally, in our experience, the product 2-[18F]fluoropyridine derivatives are often more easily separated by reverse-phase semipreparative HPLC from the analogous 2-chloropyridine precursors compared to 2-nitropyridine precursors. The chloroazabenzoxazole precursor 1 was heated with [18F]KF/K2.2.2 under microwave irradiation (140°C, 60 W, 200 s) to provide [18F]MK-3328 in high specific activity (2471 ±1389 Ci/mmol, n=25) and radiochemical purity (>98%). As expected, the desired product was easily separated from the later eluting precursor 1 by reverse-phase semipreparative HPLC. The yield of [18F]MK-3328 (14±13%, n=25) provided sufficient amounts of tracer for in vivo studies without further optimization of the reaction. Since the ¹⁸F radioisotope is incorporated as an [¹⁸F]fluoroazabenzoxazole moiety in all four radioligands, it was anticipated that similar reaction conditions would be applicable for the radio-synthesis of the other three radioligands. This was indeed the case, as [¹⁸F]AD-278 and [¹⁸F]AD-269 were produced with average yields of 40%±18% (n=4) and 35%±18% (n=4),

Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>AD plaque IC₅₀ (nM)ᵃ</th>
<th>Log D</th>
<th>P-gp transport ratio</th>
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<tr>
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<td>3.03</td>
<td>1.5</td>
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<td>PIB</td>
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<td>2.23</td>
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</table>

ᵃ Values are the mean±standard deviation (number of determinations) vs. [³H]DMAB. ND=not determined.
respectively. The synthesis of $[^{18}\text{F}]$AD-265 required deprotection of a Boc-protected secondary amine following reaction of $[^{18}\text{F}]$KF/K2.2.2 with precursor 4. Therefore, the yields were slightly lower for $[^{18}\text{F}]$AD-265, with an average yield of 10%±3% ($n=3$).

Preclinical in vivo evaluation of PET tracers for human amyloid plaque is challenging since there is no validated and generally accepted in vivo animal model. For many CNS protein targets (i.e., enzymes and receptors), there is a lower species that expresses the target with a high level of homology to the human target. If the target is present at a similar concentration in a lower species, it is often useful for preclinical in vivo evaluation of candidate PET tracers and prediction of successful imaging in humans. For instance, ligands that bind with high affinity to the human mGluR5 receptor also bind with similarly high affinity to rat and monkey mGluR5 receptors [22]. Additionally, the distribution and density of mGluR5 are similar in rat, monkey and human. Therefore, rat and monkey are good animal models for evaluation of potential clinical mGluR5 PET tracers. Unfortunately for the discovery of AD plaque tracers, rodents do not naturally develop amyloid plaques similar to what is observed in humans. Therefore, transgenic mice have been developed that accumulate humanlike amyloid plaques over time [23]. However, PET tracers successful in imaging human amyloid plaques generally do not provide a useful specific signal in vivo in these transgenic rodent amyloid plaque models [24,25]. It has been reported that the concentrations of ligand binding sites on these transgenic plaques are dramatically lower compared to native human amyloid plaques, and the transgenic rodent models are therefore a questionable model for in vivo evaluation of potential human AD plaque tracers [26]. Aged rhesus monkeys (24–30 years) produce brain amyloid plaques similar to those in humans, but typically at much lower concentrations [27]. More importantly, similar to the transgenic rodent models, the amyloid plaque ligand $[^{3}\text{H}]$PIB does not bind with high affinity to amyloid plaque in chimpanzee or rhesus monkey brains (Fig. 3) [27,28]. Therefore, these species are not good models for predicting the ability of candidate PET tracers to image human amyloid plaques in vivo.

Since a robust animal model for the in vivo imaging of amyloid plaques is not available, we chose to focus on the brain kinetics of the candidate tracers in adult rhesus monkeys as an in vivo screen to differentiate compounds with similar, promising in vitro profiles. We have found that for a variety of CNS PET tracers, the monkey has been a reliable model for predicting the brain uptake and kinetics of PET tracers in humans [29–33]. A relative comparison of the peak brain uptake and the subsequent kinetics of candidate tracers should be useful for prioritizing a lead amyloid plaque tracer candidate. We were especially interested in the tracer kinetics in white matter brain tissue. Plaque tracers are typically retained in midbrain and white matter but cleared rapidly from grey matter, such that tracer uptake beyond the early time points is dominated by midbrain and white matter uptake in healthy elderly subjects [3,34,35]. Since amyloid plaques are most abundant in the cortical grey matter, the white matter that innervates throughout the cortex could be a complicating factor in discerning specific tracer uptake in nearby grey matter regions [34]. This complication may explain why MCI patients evaluated with amyloid plaque

Fig. 3. (A) Localization of amyloid plaques by 6E10 immunohistochemistry in rhesus monkey temporal cortex. (B) Total $[^{3}\text{H}]$PIB binding in an adjacent brain slice, revealing insignificant binding of the ligand to rhesus monkey amyloid plaques.

Fig. 4. A representative template (transverse view; overlaid on MRI image) defining cortex (blue) and white matter (red) regions of interest.
PET tracers are often characterized as having either AD-like or normal-like patterns of tracer uptake rather than an intermediate pattern of uptake [36,37]. Since white matter uptake may be a major liability for amyloid plaque tracers, we felt that the unique approach of a comparative analysis of the tracer kinetics in monkey brain grey and white matter could provide us with a valuable data set for selecting an amyloid plaque PET tracer for further evaluation in human AD patients.

The strategy for drawing the key ROI (cortex, cerebellum and white matter) for the monkey PET studies is a critical consideration. It is common for white matter to be drawn as a region of interest for some CNS PET tracers. In most cases, however, the white matter region of interest (e.g., the centrum semiovale) is drawn conservatively using magnetic resonance imaging (MRI) as a guide to help avoid partial volume effects from tracer uptake in nearby grey matter regions [38]. Since we were interested in tracer uptake in

Fig. 5. Time–activity curves in selected regions of rhesus monkey brain for four potential amyloid plaque PET tracers and $[^{18}F]$AV-45. Δ=white matter; ○=cortex; □=cerebellum.
white matter and how this may influence the interpretation of tracer uptake in the cortex, we defined the ROI for white matter generously, extending it to the edges of the cortical grey matter (Fig. 4). Rather than relying solely on MRI for ROI selection, the cortex and white matter regions were defined by cluster analysis, where differences in tracer kinetics between the white matter and grey matter were utilized to help define tracer uptake in the white matter vs. tracer uptake in the cortical grey matter [18]. A similar strategy was used to define the cerebellar cortex, which was used as the reference region, and avoid spillover from tracer uptake in the cerebellar nuclei.

Baseline PET scans were acquired for each candidate PET tracer in the same rhesus monkey. The resulting TACs showed that the candidate tracers readily penetrated the BBB; peak uptake in the cerebellum was very high, ranging from 2.4 to 3.2 SUV. The TACs from the four candidate tracers are shown in Fig. 5. Peak uptake for [18F]AD-278 was later (∼15 min) relative to the other three tracers (∼5 min). For all tracers, peak tracer uptake in white matter uptake was lower than in grey matter, but as expected, the tracer uptake was retained in the white matter such that white matter uptake was higher than cortical uptake at later time points. Uptake and kinetics in grey matter regions such as the cortex and cerebellum were similar, showing rapid washout from brain tissue. This was expected since adult rhesus monkeys have very low levels of amyloid plaque deposits; and furthermore, a ligand for human amyloid plaque (PIB) has been shown to bind poorly to rhesus monkey amyloid plaque [27,28].

The key parameters of interest for the monkey PET studies were the tracer BPs in cortex and white matter. Binding potential was determined using Logan graphical analysis using the cerebellar cortex as a reference region. The cerebellar cortex was used as a reference region in humans since it is known to be relatively free of amyloid plaque deposits [34]. High BP in the white matter is of primary concern and is undesirable since, in human PET studies, it can interfere with interpretation of binding to amyloid plaques in adjacent cortical regions. Theoretically, the cortical BP in rhesus monkey should be zero since specific binding to amyloid plaques is not expected. Therefore, high BP in the cortex should primarily be a reflection of partial volume effects (i.e., signal spillover) from tracer binding in white matter and is therefore also unfavorable. For comparison, a baseline PET scan was acquired in the same rhesus monkey with [18F]AV-45. A summary of BP values in monkey R352 for the candidate tracers and [18F]AV-45 is listed in Table 2, and summed PET images are shown in Fig. 6. [18F]AD-265 had the highest BP in the cortex, which is consistent with its high white matter BP, and was clearly the least attractive PET tracer from this perspective. [18F]AD-278 showed moderate BP in the white matter, similar to [18F]AV-45. [18F]MK-3328 and [18F]AD-269 showed the most favorable kinetic profile, with the lowest BP in white matter. Compared to [18F]AV-45, the white matter BP was ∼twofold lower for [18F]MK-3328 and [18F]AD-269. Interestingly, the higher white matter BP for [18F]AV-45 and [18F]AD-278 did not have a large impact on the cortical BP, as the cortical BP for these PET tracers was similar to the cortical BP for [18F]MK-3328 and [18F]AD-269 (Table 2). A set of baseline PET scans in a second monkey (R460) was performed to further compare [18F]AD-278, [18F]MK-3328 and [18F]AD-269 since they had similar BP values in the cortex. The relative comparison of tracer BPs in monkey R460 was comparable to the BPs found in monkey R352. All three tracers had similar BP in the cortex, but [18F]AD-278 again had the highest BP in the white matter, making it a less attractive amyloid PET tracer candidate. [18F]MK-3328 and [18F]AD-269 did not show a significant difference in affinity for amyloid plaques and had similar BP values in monkey white matter and cortex. However, [18F]AD-269 was

Table 2

<table>
<thead>
<tr>
<th>PET tracer</th>
<th>BP in monkey R352</th>
<th>BP in monkey R460</th>
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<tr>
<td></td>
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<td>White matter</td>
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<tr>
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<td>[18F]AV-45</td>
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<td>0.33</td>
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Fig. 6. Summed PET images (45–90 min) in monkey R352 under propofol anesthesia for four candidate amyloid plaque PET tracers and [18F]AV-45. Images are normalized to cerebellum uptake and overlaid on an MRI image (transverse plane). The tracer uptake scale is in SUV to normalize for differences in the amount of radioactivity administered.
shown to be more lipophilic (log D=3.42) compared to [18F]MK-3328 (log D=2.91); and therefore, [18F]MK-3328 was selected as the preferred radiotracer. For CNS PET tracers, lower lipophilicity is associated with lower nondisplaceable binding in brain tissue, which in this case translates to the potential for a larger specific signal in vivo for [18F]MK-3328 in AD patients. Autoradiography studies in brain slices from AD patient brain donors comparing the binding of [3H]MK-3328 and [3H]AD-269 illustrate this principle from an in vitro perspective (Fig. 7). [3H]MK-3328 (5 nM) demonstrated clear, punctate binding to amyloid plaque deposits in the frontal cortex of a brain with amyloid pathology (Fig. 7, panel A) that can be completely blocked by 1 µM unlabeled MK-3288 (Fig. 7, panel B). In contrast, [3H]AD-269 (5 nM) did not display a punctate binding pattern (Fig. 7, panel F). The specificity of [3H]MK-3328 for amyloid plaques was further demonstrated by the lack of binding in the cerebellum of an AD patient brain donor, a brain region known to have very low levels of amyloid plaque deposits (Fig. 7, panel C). Similarly, a lack of [3H]MK-3328 binding was observed in the frontal cortex of a non-AD brain donor (Fig. 7, panels D and E).

4. Conclusion

Four radioligands were identified with attractive properties for a human amyloid plaque PET tracer: high affinity for human amyloid plaques in vitro, moderate lipophilicity and good cell permeability in a P-gp transfected cell line. A unique approach was utilized to evaluate and prioritize the candidate PET tracers: high BP in cerebral white matter is considered an unfavorable feature of amyloid plaque PET tracers, and this characteristic was evaluated in rhesus monkeys. In rhesus monkey PET studies, [18F]MK-3328 displayed high brain uptake with relatively low BP in white matter and cortical grey matter. Autoradiography studies with [3H]MK-3328 showed punctate, displaceable binding in the cortical grey matter of an AD patient brain slice, with no discernable binding in the cerebellum. [18F]MK-3328 is a promising PET tracer for in vivo quantification of amyloid plaques in humans, and investigation of [18F]MK-3328 in human healthy volunteers and AD patients is in progress.

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References