Research Article

Mapping the Target Localization and Biodistribution of Non-Radiolabeled VMAT2 Ligands in rat Brain

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Abstract. Imaging targeting vesicular monoamine transporter (VMAT2) alterations is a sensitive tool for early diagnosis of Parkinson's disease. Our group has reported several novel 2-amino-DTBZ derivatives as potential VMAT2 imaging agents. The objective of this paper is to develop a non-radiolabeled methodology to screen the candidate compounds for accelerating the drug discovery process. 9-[¹⁸F]fluoropropyl-(+)-dihydrotetrabenazine ([¹⁸F]AV-133) is a PET imaging agent targeting VMAT2 binding sites in the brain. Nonradioactive AV-133 was injected (iv) into rats, at the end of the allotted time, the animals were killed and six regions of brain and plasma from each animal were processed for quantitative measurement of AV-133 by LC-MS/MS. These data were converted to the percentage injected dose per gram tissue weight (%ID/g tissue) and the brain target tissue to background ratios to allow direct comparison with data obtained by gamma counting of the injected radioactive [¹⁸F]AV-133. The %ID/g and the brain target tissue to background ratios calculated using the LC-MS/MS method were highly correlated to the values obtained by standard radioactivity measurements of [¹⁸F]AV-133. The pattern of AV-133 in rat brain was consistent with the known distribution of VMAT2. The concordance indicated that high-sensitivity LC-MS/MS is an indispensable tool in evaluating the quantity of administered chemical in tissue as part of the development of new molecular imaging probes. Furthermore, several novel 2-amino-DTBZ derivatives were detected using this methodology, and their biodistribution data in rat brain were obtained. The information about target engagements of candidates was provided.

KEY WORDS: AV-133; biodistribution; LC-MS/MS; PET imaging agents; positron emission tomography.

INTRODUCTION

The vesicular monoamine transporter (VMAT2), which is located in the vesicle membranes in neurons, performs a secondary type of transport by storing and packaging the monoamine neurotransmitters into vesicles (1). VMAT2 allows dopamine (DA), norepinephrine (NE), epinephrine (EPI), histamine (HIS), and serotonin (5-HT) uptake into neurons and endocrine cells. A reduction of VMAT2 in brain was observed in Parkinson's disease (PD) and other neurodegenerative diseases. Because VMAT2 is less susceptible than dopamine transporter (DAT) to compensatory changes and drugs used to treat PD, some have suggested that it may be superior to DAT as a marker of dopaminergic neurons (2,3). Imaging VMAT2 alterations is a sensitive tool for early diagnosis of PD (4). Additionally, the VMAT2 is abundantly expressed in human beta cells. Radiolabeled analogues of

tetrabenazine (TBZ; a low molecular weight, cell-permeant VMAT2-selective ligand) have been employed for pancreatic islet imaging in humans (5,6).

Positron emission tomography (PET) is a very sensitive molecular imaging technique. When employed, appropriate-labeled compounds with short-lived radionuclides such as ¹¹C, ¹⁵O, ¹³N, and ¹⁸F, PET can quantitate physiological processes in a non-invasive manner (7). ¹⁸F-labeled analog of dihydrotetrabenazine (DTBZ), 9-fluoropropyl-(+)-dihydrotetrabenazine (a.k.a. [¹⁸F]AV-133) was developed as a PET imaging agent for Parkinson's disease. [¹⁸F]AV-133 displays excellent binding affinity for VMAT2 sites ($K_i=0.1$ nM) in monkey and human brain. Preliminary clinical studies suggested that [¹⁸F]AV-133 can sensitively detect monoaminergic terminal reductions in PD patients (8,9). Studies with [¹⁸F]AV-133 may allow the presymptomatic identification of individuals with disorders characterized by the degeneration of dopaminergic nigrostriatal afferents. It is currently under phase III clinical trials to establish its usefulness in the diagnosis of neurodegenerative diseases including dementia with Lewy bodies and Parkinson's disease. Recently, a series of fluoroalkyl DTBZ derivatives targeting VMAT2 binding sites in the brain have been developed. Our group has reported several novel 2-amino-DTBZ derivatives as potential VMAT2 imaging agents (Fig. 1). By substituting the amino group with an N-alkyl group and

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forming the secondary amino-DTBZ derivatives, the aim was that the ligands are expected to have a lipophilicity needed to reach the intracellular target (VMAT2) and bind with high affinity and selectivity (10). Radiosyntheses need extra radiolabeling precursors, which will double the workload of organic synthesis. Moreover, it is difficult to reduce side reactions, improve radiolabeling yields, and purify final radioactive products and so on. Therefore, the biological evaluation of these compounds cannot be performed until now.

Regional uptake and target tissue to background ratios are the key steps in the early stage of preclinical radiopharmaceutical discovery to understand target engagement. Because radiolabeling is time consuming, expensive, and technically difficult, it is useful to develop a non-radiolabeled methodology to screen the candidate compounds for accelerating the drug discovery process. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) offers high sensitivity, excellent selectivity, automation, and superior accuracy in the detection and quantitation of trace analytes (11). LC-MS/MS is an alternative approach to radiolabeled methods for measuring receptor occupancy, and it has been successfully used in quantification of non-radiolabeled ligand (tracer) for dopamine D₂, serotonin 5HT_{2A}, NK1, opiate, and histamine H₃ receptor occupancy in preclinical species (12–17). It is a promising method to speed up the candidate screening process in high-throughput fashion as well as characterization of target expression by using LC-MS/MS characterization at a preclinical stage.

In previous papers (18,19), we evaluated the effect of a pseudo-carrier on pharmacokinetics of AV-133 using a UPLC-MS/MS method, and the concentrations of AV-133 and its pharmacokinetics in the brain and blood of rats were also obtained by using microdialysis and LC-MS/MS. These results confirmed that LC-MS/MS is useful in the evaluation of radiopharmaceuticals. In this manuscript, the LC-MS/MS method was further optimized and validated; the target localization and biodistribution of non-radiolabeled AV-133 in rat brain were mapped. The differential uptake ratio (%ID/g) and the brain tissue-specific binding ratio of the non-radiolabeled VMAT2 ligand AV-133 obtained by the LC-MS/MS method matched very well with the value obtained by standard radioactivity measurements of [¹⁸F]AV-133. The pattern of AV-133 in rat brain was consistent with the known distribution of VMAT2. Furthermore, several novel 2-amino-DTBZ derivatives were evaluated, and their biodistribution data in rat brain were obtained. The methodology will be useful to speed up the preclinical candidate screening process to identify novel tracer for imaging and discovering novel medicinal entities targeting VMAT2.

EXPERIMENTAL

Chemicals and Reagents

AV-133 (purity >98%) were prepared as reported previously (9). Tetrabenazine (TBZ), used as internal standard (IS), was obtained from Dalian Meilun Biology Technology (Dalian, Liaoning, China). Methanol and acetonitrile

were of HPLC grade and purchased from Merck (Damstadt, Germany). HPLC grade ammonium acetate was purchased from J. T. Baker (Phillipsburg, NJ, USA). Distilled deionized water (18.2 MΩ) was made by an Elga Classic UVF Purification System (Buckinghamshire, UK). Other chemicals were of analytical reagent grade and purchased from commercial sources. [¹⁸F]AV-133 was prepared using a method previously reported (9,20) and the radiochemical purity was over 97%. Compounds 1, 2, and 3 were synthesized in our laboratory.

Animals

Male Sprague–Dawley rats, 6–7 weeks old, weighing 180–220 g, were obtained from Vital River Laboratory Animal Co. Ltd. (Beijing, China). Rats were kept in an environmentally controlled room (temperature=20–23°C, humidity=50±5%, 12-h light/dark cycle) for 1 week before the experiments. All the animals were maintained according to the Chinese government guidelines for care and use of laboratory animals. The rats were fasted overnight before the day of the experiment.

Preparation of Standard and Quality Control Samples

Stock solutions of AV-133 and tetrabenazine (IS) at 1.00 mg/mL were prepared in methanol, respectively. Calibration solutions were diluted with rat blank plasma/brain tissue homogenate to produce the eight concentrations of AV-133 (2.00, 10.0, 20.0, 50.0, 100, 200, 400, and 800 ng/mL), and the IS at the concentration of 200 ng/mL. The quality control (QC) solutions were similarly prepared at concentrations of 10.0, 200, and 750 ng/mL for AV-133 by separate weighing of the reference substance.

An IS solution (200 ng/mL) was also prepared by diluting the 1.00 mg/mL stock solution of tetrabenazine with methanol. Calibration standards and QC samples were stored at –20°C, and were brought to room temperature before use.

LC-MS/MS Method and Validation

The LC-MS/MS method was optimized and validated as described previously (18,19). Chromatography analysis was performed on a Waters ACQUITY module (Waters, Milford, MA, USA). The chromatographic analysis was performed on an XTerra C18 column (150 mm×2.1 mm i.d., 5-μm particle size, Waters, Milford, MA, USA) using gradient elution. The mobile phase consisted of 1-mM ammonium acetate in water (A) and 1-mM ammonium acetate in acetonitrile (B). The gradient program was set at 10% for the first 2.0 min, linearly increasing from 10%B to 80%B in the next 7.0 min, and then returning to 10%B by 11 min. The flow rate was 0.2 mL/min. The end time of the program was set at 15 min. The injection volume was 10 μL.

Mass spectrometric detection was carried out on a Micromass Quattro micro API mass spectrometer (Waters, Milford, MA) with an electrospray ionization (ESI) interface. Quantitative analysis was performed using a multiple reaction monitoring mode (MRM). In order to avoid the interference of endogenous components in tissue homogenate, internal calibration was used. Tetrabenazine (TBZ) was used as

Fig. 1. The chemical structures, chromatograms and product ion mass spectra of $[M+H]^+$ ions of **a** Compound 1, **b** Compound 2, and **c** Compound 3

internal standard (IS), the transitions for IS were m/z 318.2 $\rightarrow$ 220.3 and 318.2 $\rightarrow$ 192.3, cone voltage was 30 V, and collision energy was 20 and 25 eV. Data processing was performed using MassLynx 4.1 software.

The optimized method was validated by a set of parameters that were in compliance with the reference guidelines as defined in FDA documents (21).

Calibration Curves and Sensitivity

The linearity was assessed by assaying calibration curves in plasma/brain tissue homogenate at eight concentration levels ranging from 2.00 to 800 ng/mL. The curves were fitted by a weighted ($1/x$) least-squares linear regression method through the measurement of the peak-area ratio of the analyte to IS. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better.

Accuracy and Precision

Accuracy and precision of the method were evaluated by analyzing QC samples at three concentration levels (10.0, 200, and 750 ng/mL) in five replicates on five validation days. The accuracy was required to be within 85–115%, and the precision not to exceed 15%.

Specificity

The specificity of the method was established using a minimum of six independent sources of rat plasma/brain tissue homogenate. There should be no interference from the endogenous materials at the retention time of both AV-133 and IS.

Recovery and Matrix Effect

Recovery of the analytes from the extraction procedure was performed at 10.0, 200, and 750 ng/mL. It was evaluated by comparing the peak area of spiked samples to the peak area of unprocessed samples (quality control working solutions spiked in processed plasma/brain tissue homogenate).

To certify the extraction procedure, the *ex vivo* recovery was also assessed by injecting rats with $[^{18}\text{F}]$ AV-133 ($n=3$). The animals were sacrificed at 30-min post dose then blood samples and brain region samples were harvested and analyzed in a gamma counter (Wizard, Perkin Elmer). Then, the blood and striatum samples were processed as described above, and detected using gamma counter. The *ex*

vivo recovery was evaluated by comparing gamma counting of processed samples to the harvested samples.

Matrix effect is the suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples. To evaluate the matrix effect (ME) of AV-133 and IS, six different blank plasma/brain tissue homogenate were extracted. The corresponding peak area of each analyte in spiked plasma/brain tissue homogenate post-extraction (A) was then compared to those of the solution standards in water (B) at equivalent concentrations. The ratio $(A/B \times 100)$ is defined as the matrix factor (MF). If the MF ranged from 85% to 115%, it was concluded that there was no significant matrix effect.

Stability

QC stability experiments were performed to evaluate the analyte stability in plasma/brain tissue homogenate samples under different conditions, simulating the same conditions, which occurred during study sample analysis. QC plasma/brain tissue homogenate samples at three concentrations (10.0, 200, and 750 ng/mL) were subjected to the conditions below. Bench top stability was assessed by analyzing QC plasma/brain tissue homogenate samples left at room temperature for 4 h which was longer than the routine preparation time of the samples. Autosampler rack stability was determined by analyzing the extracted QC plasma/brain tissue homogenate samples kept in autosampler at 4°C for 24 h. Freeze–thaw stability was investigated after three freeze (–20°C)–thaw (ambient temperature) cycles. Storage stability was investigated by analyzing QC plasma/brain tissue homogenate samples after storage at –20°C for 15 days. To meet the acceptance criteria, the %bias should be within $\pm 15\%$.

Sample Preparation

Plasma samples were pretreated by protein precipitation using methanol. To each 50 μL of plasma sample, 50 μL IS working solution and 300- μL methanol were added in turn. The mixture was vortexed for 1 min and then centrifuged at $14,462 \times g$ for 5 min. The organic phase was transferred to a tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 μL of methanol and a 10- μL aliquot of the solution was injected into the LC-MS/MS system.

Three volumes of methanol were added into the weighed harvested brain region, and then homogenated the mixture using a hand homogenizer, 50- μL IS working solution was

added, and centrifuged at $14,462\times g$ for 5 min at room temperature. The organic phase was transferred to a tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 μL of methanol and a 10- μL aliquot of the solution was injected into LC-MS/MS system.

In Vivo Brain Regional Distribution Study

Regional brain distribution of AV-133 was examined using the bioanalytical method. The study was performed in male Sprague–Dawley rats ($n=15$). The rats were divided into three groups. Briefly, rats were injected with 20 $\mu\text{g/kg}$ of AV-133 via the tail vein. Rats were sacrificed at 2-, 30-, and 60-min post administration of AV-133, respectively. Blood samples (0.2 mL) were collected into heparinized tubes. As soon as possible, the blood was centrifuged for 5 min at $5,000\times g$, and plasma was obtained. Plasma (100 μL) samples were processed as described above. The brains were isolated and different brain regions (i.e., cerebellum, cortex, striatum, hippocampus, hypothalamus, and remainder of brain) were dissected, and transferred into previously weighed, prelabeled tubes. Prelabeled tubes along with brain regions were weighed immediately after dissection and frozen on dry ice until quantification of AV-133. The net weight of the tissues was obtained by the difference of post-weight and pre-weight of the tubes.

The remaining Sprague–Dawley rats were divided into three groups by weight and regional brain distribution study of [^{18}F]AV-133. [^{18}F]AV-133 (5–8 MBq, 0.1–0.2 nmol) in saline containing 10% ethanol was administered intravenously to each rat by tail vein injection was performed. The rats were sacrificed at 2, 30, and 60 min after injection, respectively. The blood samples were collected, weighed, and analyzed in a gamma counter. Activity concentrations were calculated as percentage injected dose per gram blood weight (%ID/g blood). The brains were removed and dissected. The dissected regions (striatum, hypothalamus, hippocampus, cerebellum, cortex, and remainder of brain) were weighed (0.01–0.9 g) and counted to determine the radioactivity. Activity concentrations were calculated as percentage injected dose per gram tissue weight (%ID/g tissue).

Screening Novel 2-Amino-DTBZ Derivatives

Regional brain distributions of three novel 2-amino-DTBZ derivatives were examined according to the bioanalytical method of AV-133. Briefly, three 2-amino-DTBZ derivatives were administered to male SD rats ($n=5$) by tail vein injection, respectively. The rats were sacrificed at 30 min after injection. The blood and six different brain regional samples were collected, weighed, and prepared according to the above mentioned procedure, and then analyzed by LC-MS/MS.

To confirm the affinities of the three compounds for VMAT2, *in vitro* competitive binding assays were carried out using [^{125}I]-iodovinyl-TBZ as the competing radioligand in rat striatal tissue homogenates (10).

RESULTS AND DISCUSSION

Method Optimization and Validation

An internal calibration was used, the predominant protonated precursor $[\text{M}+\text{H}]^+$ ion of IS was m/z 318.2. The most abundant ions found in the product ion mass spectra were

m/z 220.3 and 192.3 for IS. Related mass spectrometry parameters were optimized. A complete in house method validation was performed for the analysis of the samples of interest in animal plasma/brain tissue homogenate.

Linearity and Sensitivity

The calibration curves for AV-133 were linear from 2.00 to 800 ng/mL with correlation coefficient $r^2 \geq 0.99$. The mean ($\pm$ standard deviation) regression equation from replicate calibration curves from five different validation days was: $y = (23.56 \pm 7.02)x + (2.17 \pm 0.59)$ ($r^2 = 0.9966 \pm 0.0008$) for AV-133 in plasma, and $y = (2.03 \pm 0.49)x + (0.35 \pm 0.14)$ ($r^2 = 0.9970 \pm 0.0019$) for AV-133 in brain tissue homogenate, where y is the peak-area ratio of analyte to IS and x is the plasma/brain tissue homogenate concentration of analyte. The LLOQ for AV-133 was established at 2.00 ng/mL in plasma/brain tissue homogenate, which was sensitive enough for the detection of AV-133 in rats.

Accuracy and Precision

The intra- and inter-day accuracy and precision results showed that the method is accurate and precise. The accuracy and precision for intra- and inter-day at the QC samples (10.0, 200, and 750 ng/mL) were within acceptable limits.

Selectivity

Blank rat plasma/brain tissue homogenate samples were obtained from six different rats and assayed to evaluate the selectivity of the method and the detection of interferences at the retention time of AV-133 and IS. AV-133 and IS were well separated from the co-extracted material under the described chromatographic conditions at retention times of 7.81 and 10.55 min, respectively, typical MRM chromatograms of AV-133 (peak I) and IS (peak II) in rat plasma samples are shown in Fig. 2. The LC-MS/MS chromatograms of the blank plasma sample are shown in Fig. 2a. Figure 2b shows the chromatograms for the plasma samples spiked with 5.00 ng/mL AV-133 and IS. The LC-MS/MS chromatograms of AV-133 in the plasma sample obtained from a rat at 2 min after an intravenous administration of 20 $\mu\text{g/kg}$ of AV-133 are shown in Fig. 2c. The typical MRM chromatograms of AV-133 (peak I) and IS (peak II) in rat brain tissue homogenate samples are shown in Fig. 3. Figure 3a is the LC-MS/MS chromatograms of the blank brain tissue homogenate sample. The chromatograms for the brain tissue homogenate samples spiked with 5.00 ng/mL AV-133 and IS are shown in Fig. 3b. The LC-MS/MS chromatograms of AV-133 in the plasma sample obtained from a rat at 2 min after an intravenous administration of 20 $\mu\text{g/kg}$ of AV-133 are showed in Fig. 3c.

Recovery and Matrix Effect

The plasma extraction recoveries for AV-133 at 10.0, 200, and 750 ng/mL were $93.6 \pm 1.5\%$, $93.8 \pm 1.7\%$, and $96.3 \pm 0.7\%$, respectively. The plasma extraction recoveries for AV-133 at 10.0, 200, and 750 ng/mL were $85.4 \pm 2.7\%$, $107.3 \pm 0.8\%$, and $92.8 \pm 1.2\%$, respectively. The extraction recoveries for IS (200 ng/mL in plasma/brain tissue homogenate) were $94.5 \pm 1.3\%$ and $95.6 \pm 2.1\%$, respectively. The *ex vivo* recoveries were $107.3 \pm 0.9\%$ and $92.8 \pm 0.4\%$ for plasma and brain tissue, respectively. The MFs for AV-133 in brain homogenate at

Fig. 2. Representative MRM chromatograms of **a** blank plasma; **b** plasma sample spiked with 5.00 ng/mL AV-133 and IS; **c** plasma obtained from a rat at 30 min after an intravenous administration of 20 µg/kg of AV-133 I: AV-133 (m/z 366.2→348.2 and 366.2→320.3); II: IS (m/z 318.2→220.3 and 318.2→192.3)

concentrations of 10.0, 200, and 750 ng/mL were 99.7%, 95.3%, and 102.1%, respectively. The MFs for AV-133 in plasma at concentrations of 10.0, 200, and 750 ng/mL were 85.5%, 90.6%, and 101.6%, respectively. These results showed that ion suppression or enhancement from plasma/brain tissue homogenate matrix was negligible in the present condition.

Stability

The stability experiments were performed thoroughly to evaluate their stability in plasma/brain tissue homogenate

samples under different conditions. The values for the percent change for all stability experiments are 91.17–109.69%.

In Vivo Brain Regional Distribution Study

Extraction Procedure

AV-133 has a high affinity to rat brain VMAT2, which has a high density in tissues such as striatum. Normally,

Fig. 3. Representative MRM chromatograms of **a** blank brain tissue homogenate; **b** brain tissue homogenate sample spiked with 5.00 ng/mL AV-133 and IS; **c** brain tissue homogenate sample obtained from a rat at 30 min after an intravenous administration of 20 µg/kg of AV-133 I: AV-133 (m/z 366.2→348.2 and 366.2→320.3); II: IS (m/z 318.2→220.3 and 318.2→192.3)

Table I. The Biodistribution of AV-133 obtained by LC-MS/MS (mean $\pm$ SD, $n=5$) and [^{18} F]AV-133 in Male SD Rat Brain at 2-, 30-, and 60-min Post iv Injection (Mean $\pm$ SD, $n=3$)

Tissue	Time after injection(min)					
	2		30		60	
	LC-MS	Radioactivity	LC-MS	Radioactivity	LC-MS	Radioactivity
Hypothalamus	0.84 $\pm$ 0.38	0.78 $\pm$ 0.11	1.43 $\pm$ 0.19	1.28 $\pm$ 0.12	0.71 $\pm$ 0.14	0.67 $\pm$ 0.01
Hippocampus	0.49 $\pm$ 0.22	0.53 $\pm$ 0.04	0.62 $\pm$ 0.11	0.55 $\pm$ 0.04	0.25 $\pm$ 0.02	0.27 $\pm$ 0.03
Striatum	1.13 $\pm$ 0.22	1.14 $\pm$ 0.12	2.21 $\pm$ 0.41	2.49 $\pm$ 0.28	1.42 $\pm$ 0.10	1.35 $\pm$ 0.06
Cortex	0.67 $\pm$ 0.13	0.77 $\pm$ 0.13	0.50 $\pm$ 0.11	0.52 $\pm$ 0.06	0.19 $\pm$ 0.06	0.22 $\pm$ 0.03
Cerebellum	0.45 $\pm$ 0.11	0.45 $\pm$ 0.09	0.42 $\pm$ 0.19	0.46 $\pm$ 0.02	0.21 $\pm$ 0.09	0.19 $\pm$ 0.02
Remainder	0.50 $\pm$ 0.24	0.59 $\pm$ 0.10	0.62 $\pm$ 0.09	0.55 $\pm$ 0.11	0.26 $\pm$ 0.06	0.28 $\pm$ 0.02
Whole brain	0.54 $\pm$ 0.06	0.64 $\pm$ 0.09	0.63 $\pm$ 0.02	0.61 $\pm$ 0.11	0.30 $\pm$ 0.07	0.33 $\pm$ 0.03
Plasma	0.33 $\pm$ 0.07	0.26 $\pm$ 0.01	0.27 $\pm$ 0.09	0.22 $\pm$ 0.01	0.15 $\pm$ 0.04	0.10 $\pm$ 0.01

Data are means of %ID/g of tissue $\pm$ SD, $n=5$ by LC-MS and $n=3$ by radioactivity; %ID/g LC-MS liquid chromatography–mass spectrometry

radioactive analysis provided a measure of the total tissue concentrations of [^{18} F]AV-133, mainly the bound [^{18} F]AV-133 in target tissue (9,22). It is vital for brain regional distribution study by LC-MS/MS to release the bound drug. In the present study, the extraction procedure of the plasma/brain tissue homogenate samples was performed through a protein precipitation procedure using methanol. Different types of extraction procedures, including liquid–liquid extraction (LLE) with ethyl acetate, ethyl acetate–ether, n-hexane–ethyl acetate, etc., were tried during the method development, but they were limited by low or irreproducible extraction recoveries for analyte. Finally, the method of protein precipitation with methanol was optimal. The *ex vivo* recoveries also proved that the protein precipitation with methanol was available.

Dose Optimization

When high doses of AV-133 (0.1, 0.5, and 1.0 mg/kg, respectively) were injected, the concentrations of AV-133 increased were observed in various brain regions with increasing dose; however, the concentration difference of AV-133 between striatum and cerebellum regions was not obvious at the doses greater than

0.1 mg/kg. This may be caused by VMAT2 saturation kinetics at higher chemical doses. Considering the sensitivity of the analytical method, adequate cerebellum AV-133 concentration and target tissue to background ratio, a dose of 20 μ g/kg was selected. The dose was approximately 50 to 100 times more than that a no-carrier-added radioactive dose.

Brain Regional Distribution

The degree of AV-133 uptake from plasma into brain tissue was estimated from the ratio of %ID/g in brain homogenate over the plasma (%ID/g_{brain}/%ID/g_{plasma}). The AV-133 mean brain levels were found to about be 2.77, 2.46, and 3.30 at 2-, 30-, 60-min post dose, respectively. AV-133 gained ready access to the brain with higher uptake in target tissue than with other brain substructures. The brain regional distribution data were listed in Table I. Maximum uptake of AV-133 in striatum was found at 30-min post dose. At 30-min post dose, high levels of binding observed in striatum (2.21 $\pm$ 0.41%ID/g), moderate binding occurred in hypothalamus (1.43 $\pm$ 0.19%ID/g), hippocampus (0.62 $\pm$ 0.11%ID/g), and remainder of brain (0.62 $\pm$ 0.99%ID/g) with

Fig. 4. The regional brain distribution pattern of AV-133 in male SD rat brain at 30 min after an intravenous administration of 20 μ g/kg of AV-133. CB cerebellum, HT hypothalamus, HP hippocampus, ST striatum, CT cortex, RE remainder

Table II. The Target Tissue to Background Concentration Ratio of AV-133 (20 µg/kg i.v. Mean±SD, *n*=5) and [¹⁸F]AV-133 in Male SD Rats (Mean±SD, *n*=3)

Tissue	Time after injection(min)					
	2		30		60	
	AV-133	[¹⁸ F]AV-133	AV-133	[¹⁸ F]AV-133	AV-133	[¹⁸ F]AV-133
Hypothalamus	1.87±0.20	1.55±0.56	3.40±0.27	2.78±0.21	3.38±0.19	3.65±0.23
Hippocampus	1.10±0.14	1.00±0.27	1.48±0.21	1.19±0.06	1.19±0.08	1.38±0.14
Striatum	2.53±0.26	2.91±0.99	5.26±0.32	5.40±0.28	6.76±0.42	7.52±0.50
Cortex	1.50±0.13	1.50±0.54	1.19±0.12	1.13±0.09	0.90±0.09	1.11±0.17

%ID/g tissue/%ID/g cerebellum ratios for the AV-133 obtained by LC-MS/MS±SD, *n*=5; %ID/g tissue/%ID/g cerebellum ratios for [¹⁸F]AV-133 obtained by radioactivity±SD, *n*=3

much lower levels in cortex (0.50±0.11%ID/g) and cerebellum (0.42±0.19%ID/g). The regional distribution of AV-133 in rat brain was found to be, striatum > hypothalamus > hippocampus = remainder of brain > cortex > cerebellum, and is in accordance with the distribution and regional densities of VMAT2 measured using [¹⁸F]AV-133 in rat [7, 24]. The regional brain distribution pattern of AV-133 in the rat brain at 30-min dose post-injection was mapped according to the levels of concentration (Fig. 4). It can be seen that AV-133 exhibited high uptake in the brain regions expressing VMAT2 sites. Low uptakes of the compound were obtained in brain regions not expressing VMAT2. The pattern reflected the known distribution of the VMAT2 and was consistent with previous reports. A high level of uptake was observed in the striatum, moderate uptake levels in hypothalamus, hippocampus, remainder of brain and cortex. The cerebellum had the lowest uptake because it has no VMAT2; therefore, cerebellum was taken as a reflection of non-VMAT2-associated binding. AV-133 concentration ratio in striatum to cerebellum was found to be minimum at 2-min post dose, and increased gradually with time, at 30-min post dose the AV-133 concentration ratio was found to be 5.26 and maintained up to 60 min.

Comparison with Radioactivity

Biodistribution studies of [¹⁸F]AV-133 in rat brain were reproduced as previous reported (22). To avoid competing binding to VMAT2, different rats were used to perform radioactive analysis. The measured data were showed in Table I. When compared with the reference, the uptake of

[¹⁸F]AV-133 in striatum was lower than that of reference, this may be caused by difference of animal and instrument, and so on. However, as shown in Table II, the striatum to cerebellum ratios were in good agreement with the reported data, which were 2.41, 5.62, and 6.61 at 2, 30, and 60 min, respectively. Direct comparison of the %ID/g tissue determined by LC-MS/MS in each region and striatum to cerebellum ratios with data obtained by gamma counting of the [¹⁸F]AV-133, these data obtained by the two methods matched very well. The other brain regions to cerebellum uptake ratios determined by the two methods differed by less than 20%, and there were no statistic differences between radioactivity and LC-MS/MS analysis.

Biodistribution of Novel 2-Amino-DTBZ Derivatives

The brain regional distribution data of three novel 2-amino-DTBZ derivatives were listed in Table III. The results showed that the brain uptakes of three novel 2-amino-DTBZ derivatives were lower than AV-133, and the estimated uptakes from plasma in to brain tissue(%ID/g_{brain}/%ID/g_{plasma}) of compounds 1, 2, and 3 were more than 1.0; therefore, the ligands were sufficiently lipophilic to penetrate blood brain barrier (BBB). Compound 1 had higher brain uptakes than compounds 2 and 3, and the specific biodistribution of compound 1 was better than the other compounds. The striatum to cerebellum ratios of the three candidates were lower than AV-133, and the binding affinities of the compounds for VMAT2 were lower than AV-133 *in vitro*. Therefore, the three candidates have a lower affinity and selectivity for VMAT2. The *K_i* values of compounds 1, 2, and 3 obtained *in vitro* binding assays were 8.7±1.3, 7.8±3.3, and 6.7±2.6 nM, respectively. The affinities of these 2-amino-DTBZ derivatives for VMAT2 have no significant difference, and their affinities were lower than AV-133. More detailed structure–activity relationship studies are being pursued by detecting more compounds of 2-amino-dihydrotrabenazine derivatives using the non-radiolabeled methodology. Although the striatum to cerebellum ratios of the three candidates were lower than AV-133, and their brain uptakes were also lower than AV-133. Their *K_i* values were at nanomolar grade *in vitro*. The three compounds may have other potential as imaging agents targeting on beta cell mass for diabetes research. For further study, we will carry out the biodistribution of there compounds in whole animal using MS

Table III. The Biodistribution and the Striatum to Cerebellum Region Concentration Ratios of Compounds 1, 2, and 3 Obtained by LC-MS/MS (Mean±SD, *n*=5)

Tissue	Compound 1	Compound 2	Compound 3
Hypothalamus	0.32±0.02	0.16±0.01	0.09±0.01
Hippocampus	0.27±0.02	0.12±0.01	0.10±0.03
Striatum	0.29±0.04	0.13±0.03	0.09±0.01
Cortex	0.28±0.06	0.12±0.01	0.15±0.04
Cerebellum	0.13±0.04	0.08±0.01	0.05±0.01
Remainder	0.16±0.09	0.06±0.01	0.06±0.01
Whole Brain	0.17±0.01	0.08±0.01	0.07±0.01
Plasma	0.03±0.00	0.02±0.00	0.04±0.00

Data are means of %ID/g of tissue±SD, *n*=5

methods to interpret PET studies during VMAT2 ligand development.

CONCLUSIONS

A non-radiolabeled methodology that can map the target localization and biodistribution of VMAT2 ligands was developed. The brain regional biodistribution of a VMAT2 ligand of nonradioactive AV-133 was detected using a validated LC-MS/MS method, and the results were significantly concordant between the LC-MS/MS biodistribution analysis and radioactivity method in determination of %ID/g and the target tissue to background ratios in the rat brain. The pattern of AV-133 in rat brain was consistent with the known distribution of VMAT2. Furthermore, several novel 2-amino-DTBZ derivatives were screened using the non-radiolabeled methodology, and some useful biological evaluation information were obtained. The study demonstrated the availability of LC-MS/MS in determination of the target localization and biodistribution of PET imaging agents without the need to radiolabel the compounds. The non-radiolabeled approach can be an essential tool to screen new chemical entities during the early stage of radiopharmaceutical development, which may open new avenues in early development of radiopharmaceuticals.

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