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ORIGINAL ARTICLE

Synthesis and in vivo evaluation of a novel 5-HT_{1A} receptor agonist radioligand [*O*-methyl-^{11}C]2-(4-(4-(2-methoxyphenyl) piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3, 5(2*H*,4*H*)dione in nonhuman primates

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Abstract
Purpose Serotonin$_{1A}$ (5-HT_{1A}) receptors exist in high- and low-affinity states, and agonist ligands bind preferentially to the high-affinity state of the receptor and provide a measure of functional 5-HT_{1A} receptors. Although the antagonist tracers are established PET ligands in clinical studies, a successful 5-HT_{1A} receptor agonist radiotracer in living brain has not been reported. [^{11}C]MPT, our first-generation agonist radiotracer, shows in vivo specificity in baboons; however, its utility is limited owing to slow washout and immeasurable plasma free fraction. Hence we performed structure-activity relationship studies of MPT to optimize a radiotracer that will permit valid quantification of 5-HT_{1A} receptor binding. We now report the synthesis and evaluation of [^{11}C]MMP as an agonist PET tracer for 5-HT_{1A} receptors in baboons.

Methods In vitro binding assays were performed in bovine hippocampal membranes and membranes of CHO cells expressing 5-HT_{1A} receptors. [^{11}C] labeling of MMP was performed by reacting desmethyl-MMP with [^{11}C]CH_3OTf. In vivo studies were performed in baboons, and blocking studies were conducted by pretreatment with 5-HT_{1A} receptor ligands WAY-100635 and (±)-8-OH-DPAT.
Results MMP is a selective 5-HT_{1A} receptor agonist (K_i 0.15 n*M*). Radiosynthesis of [^{11}C]MMP was achieved in 30± 5% (n=15) yield at EOS with a specific activity of 2,600± 500 Ci/mmol (n=12). PET studies in baboons demonstrated specific binding of [^{11}C]MMP to 5-HT_{1A} receptor-enriched brain regions, as confirmed by blockade with WAY-100635 and (±)-8-OH-DPAT.
Conclusion We identified [^{11}C]MMP as an optimal agonist PET tracer that shows quantifiable, specific binding in vivo to 5-HT_{1A} receptors in baboons.

Keywords Brain · Imaging · Nuclear medicine · PET · Radiopharmaceuticals

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Introduction

Serotonin$_{1A}$ (5-HT_{1A}) receptors belong to the family of G-protein coupled receptors (GPCRs) and contribute to serotonin transmission in brain [1]. These receptors are localized presynaptically as somatodendritic autoreceptors in the raphe nuclei and postsynaptically in prefrontal and temporal cortex and other regions [2–7]. Serotonin$_{1A}$ receptors have been implicated in the pathophysiology of

mood and anxiety disorders, sexual function, eating disorders, neurodegenerative diseases, and the mechanism of action of antidepressants [3–11]. 5-HT_{1A} receptor agonists are being evaluated as antipsychotic drugs with fewer side-effects [12, 13]. The large population of 5-HT_{1A} receptors enables its quantification in living subjects using positron emission tomography (PET). In vitro and in vivo quantification of 5-HT_{1A} receptors reveals high receptor density in limbic regions (hippocampus, septum) and in the prefrontal and entorhinal cortices. Lower 5-HT_{1A} receptor levels are found in the thalamus, and the lowest densities are observed in the striatum, substantia nigra, and adult cerebellum [14–18].

Serotonin$_{1A}$ receptors occur in high- and low-affinity binding states. Antagonists bind to the high-affinity (HA) and low-affinity (LA) conformations of 5-HT_{1A} receptors with comparable affinity [19]. In contrast, agonists bind preferentially to the HA state of the receptor, which is coupled to G-proteins, and therefore agonists provide a measure of functional 5-HT_{1A} receptors [20, 21]. To date there is no agonist radiotracer available to study the HA binding site of 5-HT_{1A} receptors in vivo. Among the successful antagonist tracers, [^{11}C]WAY-100635 is the most commonly used 5-HT_{1A} ligand for in vivo studies in human subjects [22–24]. An agonist radioligand could provide several potential advantages over antagonist radioligands. It may: (1) enable determination of the HA:LA ratio in vivo in human brain [14]; (2) provide a measure of intrasynaptic levels of endogenous neurotransmitters [25–28]; (3) measure desensitization (downregulation) or sensitization (upregulation) of these GPCRs; and (4) provide a better estimate of receptor occupancy for agonist therapeutic agents [29]. Several efforts have been made to develop a 5-HT_{1A} receptor agonist imaging agent with limited success in vivo [30–44]. These include: the 8-OH-DPAT analogues [^{123}I]-BH-8-MeO-*N*PAT, [^{123}I]-8-OH-PIPAT, [^{11}C]OSU191, [^{11}C]LY-274601, (±)-8-OH-PPSMAT, and [^{11}C](+)S-20499 [31–36]; (*R*)-11-hydroxy-10-methylaporphine ([^{11}C]MHA) [37]; and aryl piperazines such as [^{11}C]ORG 13502, [^{18}F]FBP, [^{18}F] and [^{123}I] analogues of ORG 13063, and the [^{11}C] analogue of a thiomethane, [^{11}C](*S*)-PPMMB [38–43]. More recently, S14506 a highly selective and potent 5-HT_{1A} agonist was labeled with [^{11}C] and [^{18}F]; however, in vivo data have not been reported [44]. We have recently reported [^{11}C]-[*O*-methyl-^{11}C]2-{4-[4-(7-methoxy-naphthalen-1-yl)-piperazin-1-yl]-butyl}-4-methyl-2*H*-[1,2,4]triazine-3,5-dione ([^{11}C]MPT) as a promising 5-HT_{1A} receptor agonist radiotracer in baboons [45]. Although [^{11}C]MPT (5-HT_{1A}*K*i=1.4 n*M*, E_{max} 95% and EC_{50} 0.05 n*M*) demonstrated specific binding in 5-HT_{1A} receptor-rich regions, the slow washout in baboons made quantification of binding parameters difficult and the free fraction (percentage of parent compound not bound to plasma proteins) could not be measured. We anticipate slower washout kinetics in human subjects, further limiting the potential of [^{11}C]MPT as a 5-HT_{1A} agonist tracer. However, the promising binding specificity of [^{11}C]MPT motivated us to perform structure-activity relationship studies on MPT to develop an optimal agonist radiotracer for the in vivo imaging of 5-HT_{1A} receptors. Based on these studies, we found that phenyl analogues of MPT are excellent 5-HT_{1A} agonist ligands. The 3-methoxyphenyl analogue (MMT) of MPT possesses a 5-HT_{1A} binding affinity (K_i) of 0.7 n*M* with an EC_{50} of 0.3 n*M* and an E_{max} of 95% based on our radioligand binding and agonist-stimulated functional assays [46, 47]. Despite the attractive in vitro profile of MMT, [^{11}C]MMT did not show sufficient specific binding in our PET studies with baboons [46]. Our continuing studies led to the identification of [*O*-methyl-^{11}C]2-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5(2*H*,4*H*)dione ([^{11}C]MMP) as a superior agonist PET ligand compared with [^{11}C]MPT, and herein we report the successful synthesis and evaluation of [^{11}C]MMP in anesthetized baboons.

Materials and methods

General

The commercial chemicals used in the synthesis were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Springfield, NJ), or Lancaster (Windham, NH) and were used without further purification. ^{1}H NMR spectra were recorded on a Bruker PPX 400-MHz spectrometer. Spectra were recorded in $CDCl_3$ and chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane. The mass spectra were recorded on a JKS-HX 11UHF/HX110 HF Tandem Mass Spectrometer in the fast atom bombardment (EI+) mode. Thin-layer chromatography was performed using silica gel 60 F254 plates from E.Merck (Aston, PA). High-performance liquid chromatography (HPLC) analyses were performed using a Waters 1525 HPLC system (Milford, MA). Flash column chromatography was performed on silica gel (Fisher 200–400 mesh) using the solvent system indicated in the experimental procedure for each compound. [^{11}C]CO_2 was produced from an RDS112 cyclotron (Siemens, Knoxville, TN) and [^{11}C]CH_3OTf was synthesized in the Radioligand Laboratory of Columbia University by transferring [^{11}C]CH_3I through a glass column containing silver triflate (AgOTf) at 200°C [48]. The purities (chemical and radiochemical) and stability of [^{11}C]MMP were determined by reverse phase HPLC (RP-HPLC) (Torrance, CA) with photodiode array and sodium iodide detectors. Lipophilicity (expressed as log*P*) of [^{11}C]MMP was estimated by

determining the partition coefficient between 1-octanol and freshly prepared phosphate buffer using a standard shake flask method as described elsewhere [49]. PET studies were performed in baboon (*Papio anubis*) with an ECAT EXACT HR+ scanner (Siemens, Knoxville, TN). All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee of Columbia University Medical Center and New York State Psychiatric Institute. Metabolite analyses were performed using HPLC methods. The free fraction and metabolites were measured using a Packard Instruments Gamma Counter (Model E5005, Downers Grove, IL).

Synthesis

Synthesis of 2-(4-(4-(2-hydroxyphenyl)piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5-(2H,4H)-dione (4) A solution of 1-(2-hydroxyphenyl)piperazine (**2**, 150 mg, 0.84 mmol) and 2-(4-chlorobutyl)-4-methyl-1,2,4-triazine-3,5(2*H*,4*H*)dione (**1**, 183 mg, 0.84 mmol) in 1-butanol (5 ml) was refluxed with triethylamine (0.5 ml). After 16 h, the reaction mixture was cooled, concentrated under vacuum, and then diluted with diethyl ether (50 ml). The ether solution was washed with water and then with brine, followed by drying over anhydrous magnesium sulfate. The ether extract was filtered and evaporated, and the crude product was chromatographed over silica gel using 5% methanol in chloroform to afford desmethyl-MMP (**4**) in 61% yield (185 mg). ^{1}H NMR ($CDCl_3$) δ: 7.40 (s, 1H), 7.15 (dd,1H), 7.07 (m, 1H), 6.90 (m, 1H), 6.85 (m, 1H), 4.02 (t, 2H), 3.34 (s, 3H), 2.90 (m, 4H), 2.61 (brs, 1H), 2.45 (t, 2H), 1.82 (m, 2H), 1.57 (m, 2H); HRMS (EI^+) calculated for $C_{18}H_{26}O_3N_5$: 360.2036; Found 360.2034.

Synthesis of 2-(4-(4-(2-Methoxyphenyl)piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5-(2H,4H)-dione (5) Triethylamine (0.5 ml) was added slowly to a solution of 1-(2-methoxyphenyl)piperazine (**3**, 150 mg, 0.78 mmol) and 2-(4-chlorobutyl)-4-methyl-1,2,4-triazine-3,5(2*H*,4*H*) dione (**1**, 170 mg, 0.78 mmol) in *n*-butanol (5 ml). The reaction mixture was refluxed under argon atmosphere for the completion of the reaction and after 12 h the mixture was cooled, concentrated under vacuum, and then diluted with diethyl ether (50 ml). The ether solution was washed with water and then with brine, followed by drying over anhydrous magnesium sulfate. Ether was evaporated and MMP (**5**) was isolated from the residue by silica gel column chromatography using 5% methanol in chloroform to afford 75% yield (210 mg). ^{1}H NMR ($CDCl_3$) δ: 7.37 (s, 1H), 6.95 (m, 4H), 4.03 (t, 2H), 3.85 (s, 3H), 3.33 (s, 3H), 3.08 (brs, 4H), 2.64 (brs, 4H), 2.44 (t, 2H), 1.77 (m, 2H), 1.59 (m, 2H); HRMS (EI) calculated for $C_{19}H_{28}O_3N_5$: 374.2192; Found 374.2181.

Radiosynthesis of [O-^{11}C]-2-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5(2H,4H)-dione ([^{11}C]5] The precursor desmethyl-MMP (**4**, 0.5–1.0 mg) was dissolved in 500 μl of acetone in a capped 1-ml V-vial. Sodium hydroxide (10 μl, 5 *M*) was added and the resultant solution was allowed to stand for 2 min. High specific activity [^{11}C]CH_3OTf was transported by a stream of argon (20–30 ml/min) into the vial over approximately 5 min at room temperature. At the end of the trapping, the product mixture was diluted with 0.5 ml of acetonitrile and was directly injected into a semi-preparative RP-HPLC column (Phenomenex C18, 10×250 mm, 10 μm) and eluted with a solution of acetonitrile: 0.25 *M* sodium phosphate (35:65) at a flow rate of 8 ml/min. The product fraction with a retention time between 12 and 13 min based on γ-detector was collected, diluted with 100 ml of deionized water, passed through a classic C-18 Sep-Pak®, cartridge and washed with 10 ml water. The yield of formation of [^{11}C]MMP ([^{11}C]**5**) is typically in the range of 100–250 mCi and was eluted from Sep-Pak® using 1 ml absolute ethanol in 30% yield, based on $^{11}CH_3$OTf at end of synthesis (EOS). A portion of the ethanol solution was analyzed by analytical RP-HPLC column using UV and γ-detectors (Phenomenex, Prodigy ODS(3) 4.6×250 mm, 5 μm; mobile phase: acetonitrile:0.25 *M* sodium phosphate solution (40:60), flow rate: 2 ml/min, retention time: 6 min, wavelength: 254 nm) to determine the specific activity and purities. The [^{11}C]MMP is first diluted to a volume of 10 ml with saline and filtered through a sterile environment, and only a portion of this solution is formulated for injection. The final dosage (5±0.5 mCi) contains 50–100 μl of ethanol in 10 ml saline prepared aseptically. The formulation of [^{11}C]MMP was also analyzed by RP-HPLC to confirm the purities and specific activity and to obtain stability measurements.

Partition coefficient measurement

The partition coefficient ($\log P_{o/w}$) of [^{11}C]MMP was measured by mixing 0.1 ml of the radioligand formulation with 5 g each of 1-octanol and freshly prepared PBS buffer pH=7.4) in a culture tube [49]. The culture tube was shaken mechanically for 5 min followed by centrifugation (5 min). Radioactivity per 0.5 g each of 1-octanol and aqueous layer was measured using a well counter. The partition coefficient was determined by calculating the ratio of counts/g of 1-octanol to that of buffer. 1-Octanol fractions were repeatedly portioned with fresh buffer to get consistent values for partition coefficient. All the experimental measurements were performed in triplicate.

In vitro binding assay of MMP

Membranes from bovine hippocampus expressing the 5-HT_{1A} receptor at a density of 1.6 pmol/mg protein were prepared [50]. Incubation medium for the radioligand binding consisted of 50 m*M* TRIS-HCl (pH 7.4), 1.0 n*M* of [^{3}H]8-OH-DPAT and 30 μg membrane protein in the presence of different concentrations of MMP. The assay mixture was incubated for 60 min at 25°C. The reaction was terminated by rapid vacuum filtration onto glass fibre filters. Radioactivity trapped onto the filters was determined and compared with control values in order to ascertain any interactions of test compound with the 5-HT_{1A} binding site. Nonspecific binding was determined using 10 μ*M* of serotonin (5-HT).

Agonist-stimulated [^{35}S]GTPγS binding of MMP

These experiments were carried out as described previously with some modification [51]. Chinese hamster ovary (CHO) cells expressing 5-HT_{1A} receptors (CHO-h5-HT_{1A}) membranes (30 μg) were preincubated with MMP for 5 min at room temperature with indicated concentrations in a buffer containing 20 m*M* HEPES pH 7.4, 3 m*M* $MgCl_2$, 100 m*M* NaCl, and 3 μ*M* GDP in a final volume of 0.5 ml. [^{35}S]GTPγS (0.1 n*M*; 1250 Ci/mmol Perkin Elmer Life Science, Boston, MA) was added and the incubation was continued for 60 min at room temperature. Experiments were terminated by rapid filtration through Whatman GF/B filters followed by three washes with ice-cold 20 m*M* HEPES buffer, pH 7.4, using a cell harvester (Brandel, M-24R, Gaithersburg, MD). Bound radioactivity was determined by liquid scintillation spectrometry (Beckman, LS9000).

PET studies in baboons

Fasted animals were induced with ketamine (10 mg/kg, i.m.) and anesthetized with 1.5–2.0% isoflurane via an endotracheal tube. Core temperature was kept constant at 37°C with a heated water blanket. An i.v. infusion line with 0.9% NaCl was maintained during the experiment and used for hydration and radiotracer injection. An arterial line was placed for obtaining arterial samples for the input function. After a 10-min transmission scan, 5±0.5 mCi of [^{11}C]MMP (S.A. of 2600±500 Ci/mmol) was injected as an i.v. bolus and emission data were collected for 120 min in 3-D mode in a Siemens ECAT EXACT HR+ (CPS/Knoxville, TN). The head was positioned at the center of the field of view as defined by imbedded laser lines. Regions of interest drawn on the animal's MRI scan were transferred to co-registered automated image registration frames of PET data [52]. Blocking studies were performed by pretreatment with WAY-100635 (maleate salt, 0.5 mg/kg i.v.) and (±)-8-OH-DPAT (HBr salt, 2 mg/kg i.v.) in 12 ml saline, 30 min prior to the baseline scan.

Protein binding and metabolite analyses

The protein binding of [^{11}C]MMP in human and baboon blood samples were determined as described elsewhere [53]. The percentage of radioactivity in plasma as unchanged [^{11}C]MMP was determined by HPLC. The HPLC system consisted of a pump (Renin), and an injector equipped with a sensitive γ-detector. The injection volume loaded on the HPLC column was 10 μl–1 ml. Blood samples were taken at 2, 6, 12, 30, 60, and 90 min after radioactivity injection for metabolite analysis. The supernatant liquid obtained after centrifugation of the blood sample at 2,000 rpm for 1 min was transferred (0.5 ml) into a tube and mixed with acetonitrile (0.7 ml). The resulting mixture was vortexed for 10 s, and centrifuged at 14,000 rpm for 4 min. The supernatant liquid (1 ml) was removed and the radioactivity was measured in a well-counter and the majority (0.8 ml) was subsequently injected onto the HPLC column [column: Phenomenex, Prodigy ODS (3) 4.6×250 mm, 5 μm; mobile phase: acetonitrile:0.25 *M* sodium phosphate solution (40:60), flow rate: 2 ml/min, retention time: 6 min] connected to a Waters guard column (Resolve™ 10 μm, 90 A°) equipped with a radioactivity detector. The metabolite and free fractions were collected using a Bioscan gamma detector. All the acquired data were then subjected to correction for background radioactivity and physical decay to calculate the percentage of the parent compound in the plasma at different time points. In order to reaffirm that the retention time of the parent had not shifted during the course of the metabolite analysis, a quality control sample of [^{11}C]MMP was injected at the beginning and the end of the study. The percentage of radioactive parent obtained was used for the measurement of metabolite-corrected arterial input functions.

Results

Chemistry and radiochemistry

The synthetic route for the preparation of MMP and [^{11}C]MMP is shown in Fig. 1. Syntheses of 2-(4-chlorobutyl)-4-methyl-1,2,4-triazine-3,5(2*H*,4*H*)dione (**1**) [45], the common intermediate for the preparation of standard MMP (**5**) and radiolabeling precursor desmethyl-MMP (**4**), were achieved from 6-azaurazil in three steps. Coupling of compound **1** with commercial 2-(piperazin-1-yl)phenol (**2**) provided desmethyl-MMP (**4**) in 61% yield. Under

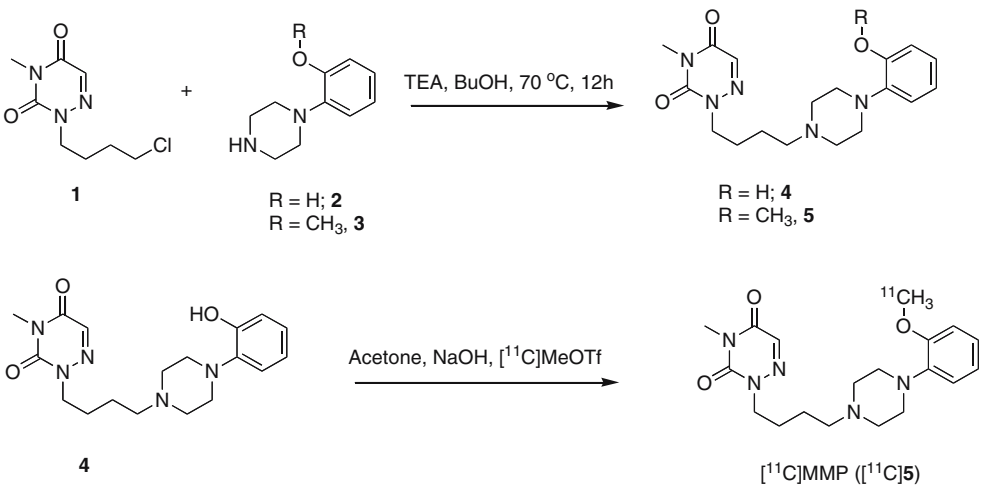

Fig. 1 Scheme for the synthesis of 2-(4-(4-(2-methoxyphenyl) piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5(2*H*,4*H*)dione (**5**), 2-(4-(4-(2-hydroxyphenyl) piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5(2*H*,4*H*)dione (**4**), and the radiosynthesis of [^{11}C]MMP

identical conditions, coupling of compound **1** with 1-(2-methoxyphenyl)piperazine (**3**) afforded the standard MMP (**5**) in 75% yield. Radiosynthesis of [^{11}C]MMP ([^{11}C]**5**) was performed by [^{11}C]methylation of the phenolate of desmethyl-MMP using [^{11}C]MeOTf, adopting a standard radiolabeling procedure for phenols (Fig. 1). The crude product was purified by RP-HPLC followed by C-18 Sep-Pak® purification to obtain [^{11}C]MMP in 30% yield at EOS (n=15, SD ±5) (Fig. 2). The identity of [^{11}C]MMP was confirmed by co-injecting the [^{11}C]-product with nonradioactive compound and comparing the HPLC retention times of the two compounds. Specific activity obtained for [^{11}C]MMP was 2,600±500 Ci/mmol (n=12) based on a standard mass curve. The total time required for the radiolabeling process was 30 min at EOS. The stability of [^{11}C]MMP formulation used for in vivo studies was analyzed at 60 min and 90 min using analytical HPLC. Column efficiency and chemical and radiochemical purities were found to be >98%, indicating that [^{11}C]MMP is stable in vitro. The log$P_{o/w}$ of [^{11}C]MMP from the shake flask method was found to be 1.1.

In vitro binding

Competition binding studies with [^{3}H]8-OH-DPAT in bovine hippocampal membranes determined the binding affinity value (K_i) of MMP as 0.15 n*M*. The affinity of MMP for various biogenic amines, brain receptors, and transporters was determined (Table 1) through National Institute of Mental Health–Psychoactive Drug Screening Program (NIMH-PDSP). MMP has a K_i of 6.75±0.6 n*M* to α_1 receptors, which is 45 times higher than 5-HT$_{1A}$ receptor affinity. MMP also exhibits nanomolar affinity to 5-HT$_7$ (K_i=12.9±3 n*M*) and D$_4$ (K_i=21.8±2 n*M*) receptors. The number of 5-HT$_7$ and D$_4$ receptors is too low in brain to be detected using PET [54–56]. The next lowest

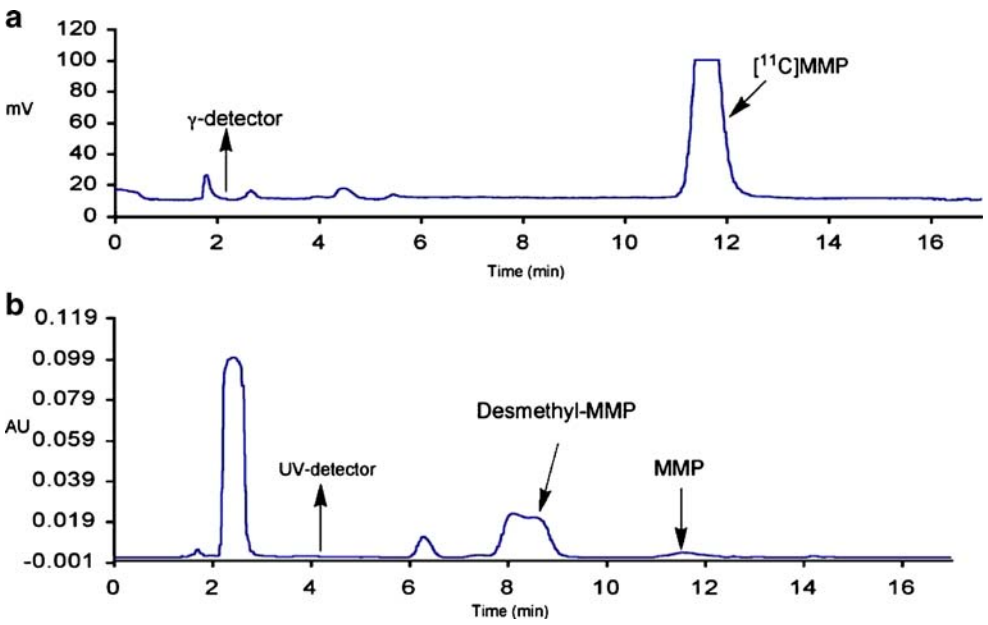

Fig. 2 HPLC chromatograms of [^{11}C]MMP: **a** radiochromatogram; **b** UV chromatogram

Table 1 In vitro binding data (K_i) of MMP

Targets	*Ki* (n*M*)	Targets	K_i (n*M*)
5-HT_{1A}	0.15±0.05	D_1, D_2	>10,000
5-HT_{1B}	643±236	D_3	>10,000
5-HT_{1D}	88.3±10.1	KAR	>10,000
5-HT_{1E}	>10,000	D_4	21.8±2
5-HT_{2A}	4,975	D_5	>10,000
5-HT_{2B}	73.6±23.4	DAT	>10,000
5-$HT_{2C,\ 3}$	>10,000	DOR	>10,000
5-$HT_{6,\ 5A}$	>10,000	EP	>10,000
5-HT_7	12.9±3	GABA	>10,000
A	>10,000	H_1	1,030
α_1	6.75±0.6	H_2	2,126
β	>10,000	H_3, H_4	>10,000
α_{2A}	96±28.5	HERG	>10,000
α_{2B}	238±35	KOR	>10,000
α_{2C}	15.4±3.1	M	>10,000
BZP	>10,000	MDR1	>10,000
Ca^+ channel	>10,000	MOR	>10,000
AMPA	>10,000	mGluR	>10,000
NET	>10,000	NMDA	>10,000
NK	>10,000	SERT	>10,000
$Sigma_1$	59.1±13.5	$Sigma_2$	123±35
V_1, V_2, V_3	>10,000	VMAT1,1	>10,000
Na^+ channel	>10,000	NT1	>10,000
CB_1, CB_2	>10,000	Imidazoline	>10,000

A adenosine, *BZP* benzodiazepine, *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, *V* vasopressin, *CB* cannabinoid, *D* dopamine, *DAT* dopamine transporters, *DOR* δ-opioid receptors, *EP* prostanoid receptors, *GABA* γ-aminobutyric acid, *H* histamine, *hERG* human ether-a-go-go, *KOR* κ-opioid receptors, *M* muscarinic, *MDR* multidrug resistance, *MOR* μ-opioid receptor, *mGluR* metabotropic glutamate receptors, *NMDAN*-methyl-D-aspartic acid, *NK* neurokinin, *SERT* serotonin transporter, *VMAT* vesicular monoamine transporter, *NET* norepinephrine transporter, *NT* neurotrophin

affinities of MMP for other tested targets were 400 times or more than 5-HT_{1A} receptor affinity [$sigma_1$ (K_i=60±13.5 n*M*), 5-HT_{2B} (K_i=73.6±23 n*M*), 5-HT_{1D} (K_i=88.3±10 n*M*), and $sigma_2$ (K_i=123±35 n*M*)]. The K_is for various other brain receptors and transporters was low (0.1–10 μ*M*). Agonist properties of MMP on 5-HT_{1A} receptors were evaluated using [^{35}S]GTPγS binding in membranes of CHO cells stably expressing the human 5-HT_{1A} receptors. MMP produced a dose-dependent increase in [^{35}S]GTPγS binding. Maximal MMP-stimulated [^{35}S]GTPγS binding (E_{max}) was 80% of that seen with 5-HT. The EC_{50} of 0.1 n*M* for MMP was comparable to that for 5-HT (0.56 n*M*); results are summarized in Fig. 3.

PET studies in baboons

PET studies in anesthetized baboon (*Papio anubis*) showed that [^{11}C]MMP penetrated the blood-brain barrier (BBB) and was retained in 5-HT_{1A} receptor-rich areas such as hippocampus, insula, cingulate, prefrontal cortex, and amygdala, whereas the thalamus exhibited lower binding and cerebellum had the least amount of radiotracer uptake (Figs. 4, 5). The distribution of [^{11}C]MMP in baboon brain is in good agreement with the known 5-HT_{1A} receptor distribution [14, 17, 45]. As can be seen from the time-activity curves (TACs) (Fig. 5),

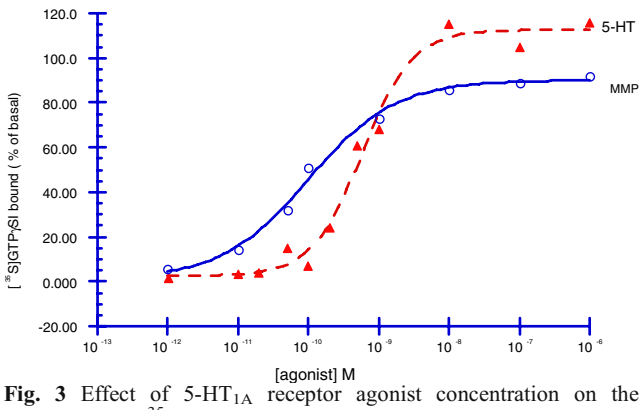

Fig. 3 Effect of 5-HT_{1A} receptor agonist concentration on the stimulation of [^{35}S]GTPγS binding in CHO cells. Values are shown as expressed as a percentage above basal, which is the binding of [^{35}S]GTPγS in the absence of agonists. Data points are means of duplicate determinations from representative experiments repeated on at least three independent occasions with similar results

the radioactivity in all regions reached a peak by 3–13 min post injection and a rapid clearance was observed for cerebellum. The binding ratios of hippocampus, insula, cingulate, prefrontal cortex, amygdala, dorsal raphe nucleus, and thalamus to cerebellum were 6.25, 5.25, 4.75, 4.0, 3.75, 3.0, and 2.0 at 105 min. The specificity of the ligand uptake was determined by blocking studies with the 5-HT_{1A} receptor antagonist WAY-100635 (0.25 mg/kg i.v.) and agonist (±)-8-OH-DPAT (2 mg/kg i.v.) 30 min prior to the administration of [^{11}C]MMP (Figs. 4, 6).

Protein binding and metabolite analyses

[^{11}C]MMP showed 41±4% (n=12) and 63±7% (n=12) protein binding in baboon and human blood samples respectively. HPLC analyses of the plasma samples (12 independent experiments) indicated only polar metabolites and the percentage of unmetabolized [^{11}C]MMP was 87.5±1.2% of total plasma radioactivity at 2 min, 84.0±2.2% at 4 min, 62.7±3.5% at 12 min, 43.1±3.7% at 30 min, 31.9±1.8% at 60 min and 23.7±3.2% at 90 min (Fig. 7). No significant change in the metabolism of [^{11}C]MMP was observed during the blockade studies.

Discussion

We have previously reported that [^{11}C]MPT is a specific 5-HT_{1A} agonist PET tracer in baboons, but the TACs of the PET images demonstrated a slow washout suggesting challenging quantification [45]. Thus, we performed structure-activity relationship studies of MPT and identified that its methoxy-phenyl analogues are candidate PET ligands for 5-HT_{1A} receptors owing to their excellent 5-HT_{1A} affinity, selectivity, agonist properties, and appropriate lipophilicity for penetration of the BBB [45–47] Even though the 3-methoxyphenyl analogue MMT has an excellent in vitro profile, it failed owing to low specific binding [47]. We therefore examined the potential of the 2-methoxyphenyl analogue MMP as a PET ligand. MMP and its precursor were synthesized from 2-(4-chlorobutyl)-4-

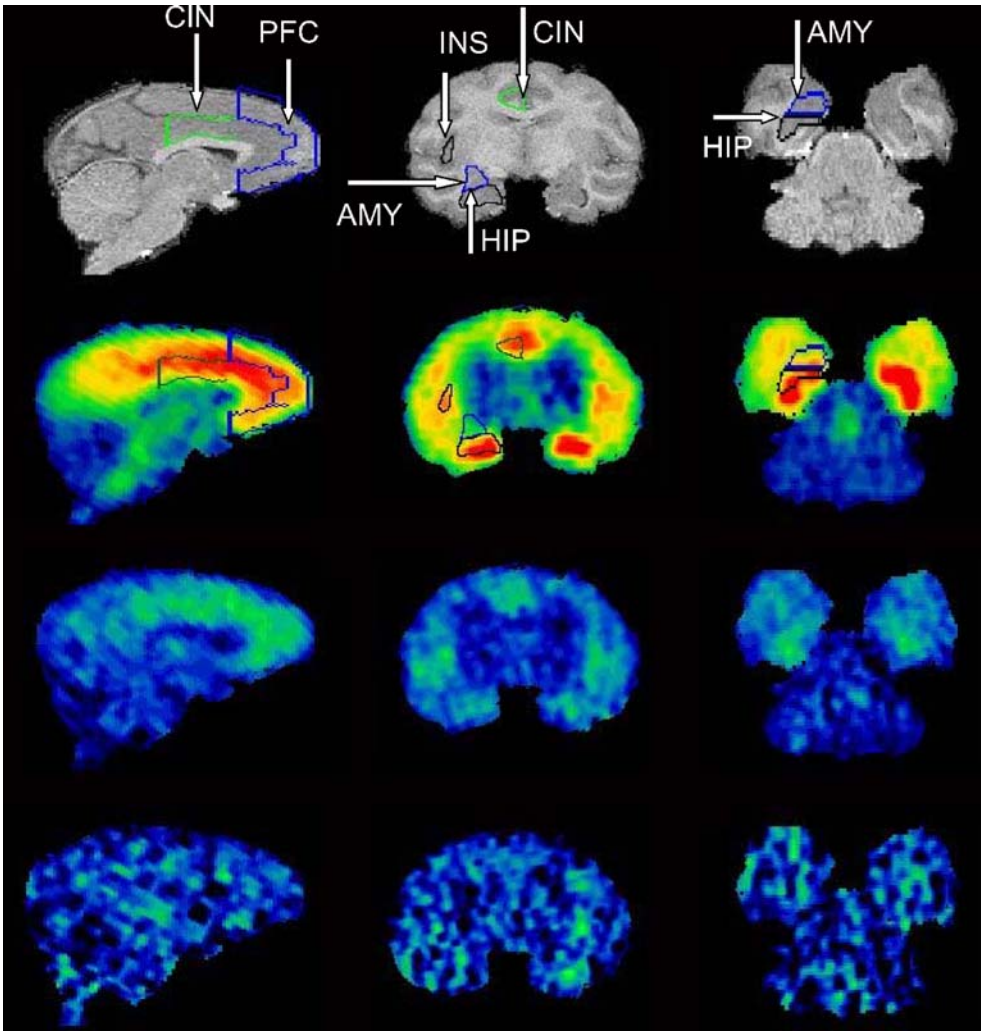

Fig. 4 MRI in baboon brain and sum of the PET images of [^{11}C]MMP activity in a baboon brain 60–120 min after the radioligand injection. *First row*: MRI; *second row*: baseline scan; *third row*: pretreatment with WAY-100635 (0.5 mg/kg i.v.); *fourth row*: pretreatment with (±)-8-OH-DPAT (2 mg/kg i.v.). *First column*: sagittal view; *middle column*: coronal view; last column: axial view. *CIN* cingulate, *PFC* prefrontal cortex, *INS* insula, *AMY* amygdala, *HIP* hippocampus

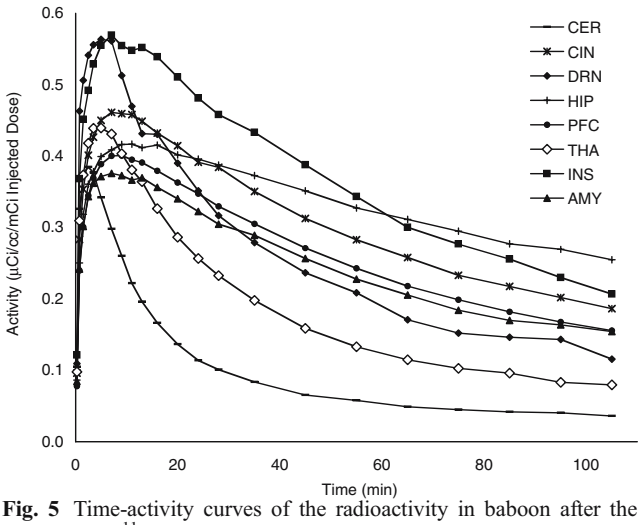

Fig. 5 Time-activity curves of the radioactivity in baboon after the injection of [^{11}C]MMP. *AMY* amygdala, *CIN* cingulate, *CER* cerebellum, *DRN* dorsal raphe nucleus, *HIP* hippocampus, *INS* insula, *PFC* prefrontal cortex, *THA* thalamus

methyl-1,2,4-triazine-3,5(2*H*,4*H*)dione in good yield using an analogous procedure to that reported for MPT (Fig. 1) [45]. The binding affinity assay in bovine hippocampal membrane shows that MMP has high (0.15 n*M*) affinity (K_i) for 5HT$_{1A}$ receptors (Table 1), and has 1:45, 1:86 and 1:145 ratios to the affinities for α_1, 5-HT$_7$ and D$_4$ receptors, respectively, with no significant affinity for other studied brain receptors, enzymes, transporters, or biogenic amines. Agonist-stimulated GTPγS binding assay confirmed that MMP is a 5-HT$_{1A}$ receptor agonist ligand with an EC_{50} slightly lower than that of 5-HT and an E_{max} value comparable to that of 5-HT (Fig. 3). Radiosynthesis of [^{11}C]MMP was achieved from the phenolate salt of desmethyl-MMP in >99% chemical and radiochemical purities, and with a specific activity at EOS (2,600± 500 Ci/mmol) (Figs. 1, 2). The radioligand was found to be stable in vitro, and in vivo PET studies in anesthetized baboon indicated high uptake of [^{11}C]MMP in brain regions known to be rich in 5-HT$_{1A}$ receptors (Fig. 4). PET images of baboon brain obtained after pretreatment with WAY-100635 and (±)-8-OH-DPAT confirmed the specificity of [^{11}C]MMP binding to 5-HT$_{1A}$ receptors (Fig. 4). TACs in Fig. 5 indicate that [^{11}C]MMP achieved an equilibrium and specific binding in 5-HT$_{1A}$ receptor-enriched brain regions.

The binding ratios of [^{11}C]MMP in various brain regions to cerebellum were found to be higher than those of [^{11}C]MPT. For example, [^{11}C]MMP had hippocampus to cerebellum and cingulate to cerebellum binding ratios of 6.25 and 4.75 at 105 min, compared with 2.9 and 2.5 for [^{11}C]MPT. Moreover, the washout of [^{11}C]MMP was faster than that of [^{11}C]MPT. The TACs at 35 and 105 min demonstrated a 30–50% faster washout for [^{11}C]MMP in almost all brain regions except cerebellum, where the washout rates reflect nonspecific binding and, as expected, are comparable to those of [^{11}C]MPT [45]. TACs of the blocking studies showed specific binding (Fig. 6) of [^{11}C]MMP to 5-HT$_{1A}$ receptors. Blocking of [^{11}C]MMP binding by the agonist (±)-8-OH-DPAT indicates the possibility of measuring the HA state of 5-HT$_{1A}$ receptors. The blockade effect of WAY-100635 is higher (~25%) for hippocampus, cingulate, and prefrontal cortex. Nonspecific or cerebellar binding was comparable for the two tracers. The lower blockade of [^{11}C]MMP binding by (±)-8-OH-DPAT compared with WAY-100635 (clog*P*=2.66±0.50) may have been due to (±)-8-OH-DPAT's (clog*P*=4.25±0.25) poor

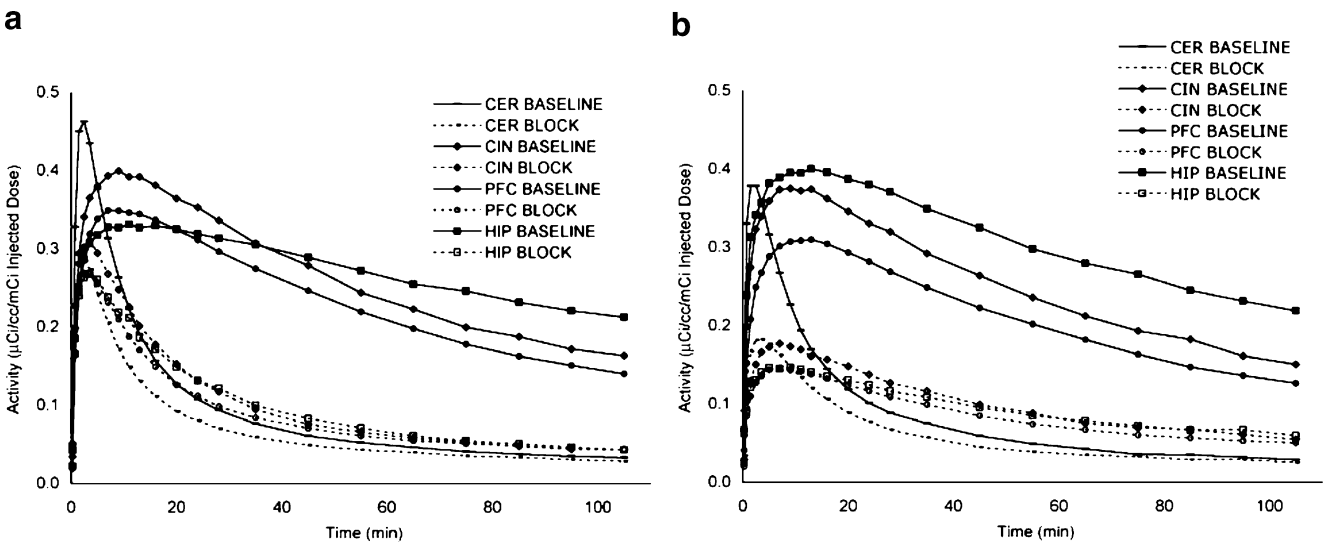

Fig. 6 Time-activity curves of the radioactivity in baboon after the injection of [^{11}C]MMP. **a** Pretreatment with WAY-100635 (0.5 mg/kg i.v.). **b** Pretreatment with (±)-8-OH-DPAT (2 mg/kg i.v). *CIN* cingulate, *CER* cerebellum, *HIP* hippocampus, *PFC* prefrontal cortex

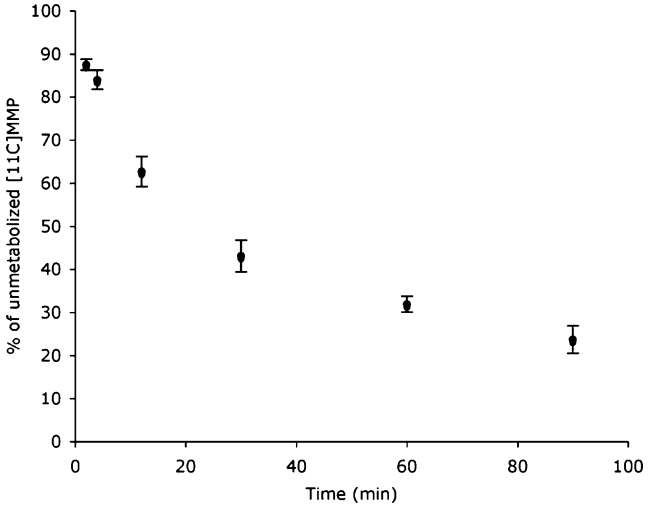

Fig. 7 Unmetabolized parent fraction of [^{11}C]MMP in baboon plasma. *Filled circles* represent the mean fraction in 12 determinations. *Error bars* are standard deviations

BBB penetration (calculated with ACD/log*P* DB program); hence sufficient (±)-8-OH-DPAT may not be occupying 5-HT_{1A} receptors to compete with [^{11}C]MMP. The minor blockade effect observed in cerebellum (reference region) was likely due to the presence of a small number of 5-HT_{1A} receptors in cerebellum [17, 45]. Fast metabolism of [^{11}C]MMP was observed in baboon plasma and the radioactive metabolites were found to be polar, suggesting an inability to penetrate the BBB. No significant change in metabolite levels was observed in the blockade studies. There was a measurable free fraction of [^{11}C]MMP in plasma: 59±6% (n=12) and 37±3% (n=12) for baboon and human plasma respectively. Therefore, in addition to the more favorable kinetics of this tracer, it also has the advantage of a measurable free fraction that will allow calculation of binding potential (B_{max}/K_d). These data suggest that in vivo binding of [^{11}C]MMP is superior to that of [^{11}C]MPT and [^{11}C]MMT, our previously identified 5-HT_{1A} agonist tracers.

Conclusion

Radiosynthesis of [^{11}C]MMP, a high-affinity and selective 5-HT_{1A} receptor agonist ligand, was developed with high purity and specific activity. PET studies in anesthetized baboon indicated selective localization of radiotracer binding to 5-HT_{1A} receptor-rich brain regions. The specificity of radioligand binding was demonstrated by blocking studies with the 5-HT_{1A} receptor antagonist WAY-100635 and the agonist (±)-8-OH-DPAT. The combination of rapid washout and specific binding with excellent target to nontarget ratios means that [^{11}C]MMP has potential as an agonist PET ligand for the in vivo quantification of 5-HT_{1A} receptors in human subjects.

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