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Synthesis And In Vivo Evaluation Of 3-[^{11}C]Methyl-(3-methoxy-naphthalen)-2-yl-(1-Benzyl-piperidin)-4-yl-Acetate (SB-235753), as a putative Dopamine D_4 Receptors Antagonist for PET

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SUMMARY

(3-Methoxy-naphthalen)-2-yl-(1-benzyl-piperidin)-4-yl-acetate (SB-235753) was labelled with ^{11}C ($t_{1/2}$= 20.4 min) as a putative radioligand for the non-invasive assessment of Dopamine D_4 receptors *in vivo* with positron emission tomography (PET).

The precursor for the radiosynthesis 3-hydroxynaphthyl-2-[(N-benzyl)-piperidyl]-acetate hydrochloride was prepared by a four-step synthesis starting from ethyl-4-pyridyl acetate. The radiolabelling consisted of methylation with [^{11}C]methyltriflate in dimethylformamide in the presence of potassium hydroxide. [^{11}C]SB-235753, was synthesised in 30 min with a radiochemical yield of 10 ± 5% (EOS, non-decay

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corrected) with 99% radiochemical purity and specific radioactivity of 10 ± 3 Ci/μmol.

Biodistribution studies in rats with [^{11}C]SB-235753 showed the uniform distribution of the tracer within different areas of the murine brain. At 30 min after injection 99% of the radioligand in plasma and 100% in cerebellum was metabolised. These findings suggest that [^{11}C]SB-235753 can not be a suitable tracer for dopamine D_4 receptor studies with PET.

KEY WORDS: SB-235753; rat, biodistribution; Dopamine D_4 antagonist; [^{11}Cmethyltriflate; *O*-methylation; 3-[^{11}C]methyl-(3-methoxy-naphthalen)-2-yl-(1-benzyl-piperidin-4-yl)-acetate, PET.

Running Title: Dopamine D_4 radioligand for PET

INTRODUCTION

The investigation of neurotransmission systems with Positron Emission Tomography (PET) is an important tool for understanding the neurochemical bases of neuropsychiatric diseases. Diversity of dopamine receptors in the brain imply the need for selective radiotracers in order to delineate the role of each receptor subtype. In recent years advances in molecular biology techniques led to the cloning of a number of different subtypes of dopamine receptors (D_1-D_5). Cloning of the dopamine D_4 gene (1) stimulated research on the synthesis of new ligands for this receptor system.

Several ligands with high affinity to dopamine D_4 receptors such as isoxazole (2), 7-azaindole (3), 2-naphthoate (4), benzamide (5), pyrrole (6), pyrrolo[2,3b]pyridine (7), benzo[g]quinoline derivatives (8) have been synthesised and some were labelled with positron emitters for studies in rodents

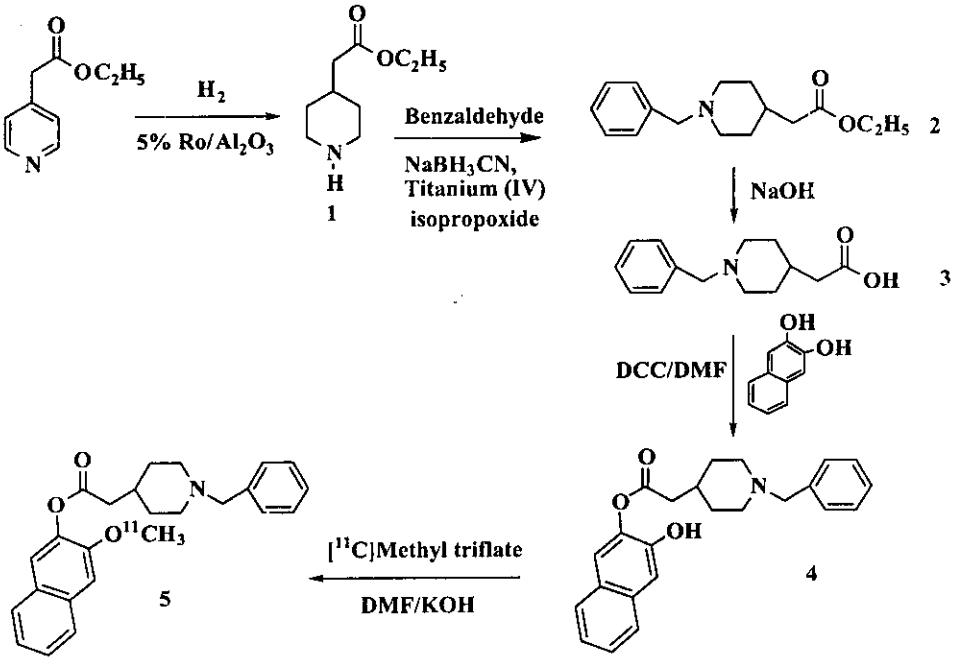
(3) and non-human primates (9). However, a tracer suitable for measuring of dopamine D_4 receptor density in humans with PET has not yet been found; the density of these receptors has been calculated so far as the difference between [^{3}H]raclopride and [^{3}H]emonapride binding (10,11). The aim of this study is the development of a PET tracer for dopamine D_4 receptors and the evaluation of its biodistribution in rats.

SB-235753, (3-methoxy-naphthalen)-2-yl-(1-benzyl-piperidin-4-yl)-acetate, was reported to possess high affinity to D_4 receptors (pKi= 8.2) and remarkable selectivity (>1000-fold) against D_2 receptor (4). This ligand has been proposed as a tool for the *in vitro* determination of the distribution and function of dopamine D_4 receptors in the brain (4). We labelled it with ^{11}C in order to investigate its pharmacological profile as a possible candidate for PET studies.

RESULTS AND DISCUSSION

Chemical synthesis

The *des*-methyl precursor **4** was prepared following the originally published procedure for SB-235753 synthesis (4) with subsequent modifications. 1-Benzylpiperidin-4-yl-acetic acid **3** was obtained by hydrogenation of pyridin-4-yl ethylacetate (12) followed by reductive benzylation of ethylpiperidyl acetate **1** in the presence of titanium(IV)*iso*-propoxide and sodium cyanoborohydride (13) and subsequent basic hydrolysis of the ethyl ester **2**. Coupling of the 2,3-dihydroxynaphtalin with 1-benzylpiperidin-4-yl-acetic acid **3** in the presence of N-N'-dicyclohexylcarbodiimide in dimethylformamide gave the *des*-methyl SB-235753 **4** in 33% yield. Esterification of benzylpiperidylacetic acid **3** required addition of molecular sieves in order to shift equilibrium towards the final product and did not involve the second hydroxyl moiety on 2,3-dihydroxynaphtalin as demonstrated by NMR analysis, which endorsed the absence of the bis-benzylpiperidyl acetate in the reaction mixture (scheme 1).



Scheme 1: Synthesis of precursor and labelling of [^{11}C]SB-235753

The best radiochemical yield of [^{11}C]SB-235753 (15% EOS) was obtained with [^{11}C]methyltriflate in dimethylformamide at -15°C in the presence of >potassium hydroxide. [^{11}C]*O*-Methylation was initially attempted with [^{11}C]methyl iodide in different solvent-base mixtures such as acetonitrile, acetone, dimethylformamide with tetrabutylammonium hydroxide (60% solution in water), potassium *tert*-butoxide or N,N-diisopropylethylamine at various temperatures ranging from -15° to 100°C according with our previous developed [^{11}C]-labelling procedures (85% radiochemical yield at 40°C using potassium *tert*-butoxide)(14). However, under these conditions the rate of incorporation of [^{11}C]methyl iodide was about 17% when we used acetone as solvent in the presence of tetrabutylammonium hydroxide and no reaction was detectable using potassium *tert*-butoxide. The major unidentified radioactive by-products could be

probably attributed to the breakdown of the title compound due to the basic hydrolysis of benzylpiperidyl acetate. Best among the tested conditions was [^{11}C]*O*-Methylation reaction using [^{11}C]methyltriflate at -15°C and potassium hydroxide as the base (45% yield) (Table 1).

Table 1. Labelling of *des*-methyl SB-235753, 1 mg of precursor dissolved in 100 µl of the solvent, 3 min reaction time.

^{11}C-Precursor	Base	Amount µmol	Temperature °C	Solvent	[^{11}C]SB-235753 %	Other ^{11}C side-products	[^{11}C]MeOH %
MeI	TBAH	8	50	Acetone	17	70	13
MeI	TBAH	3	50	DMF	0	60	40
MeTf	no base	-	25	DMF	0	66	34
MeTf	KOH	10	-15	DMF	45	22	33
MeTf	KOH	10	50	DMF	28	58	14
MeTf	TBAH	4	-15	CH_3CN	11	-	-
MeTf	TBAH	4	-15	Acetone	37	58	5
MeTf	TBAH	4	25	Acetone	15	59	26
MeTf	TBAH	8	50	Acetone	17	56	37

Since radiochemical yield obtained with [^{11}C]methyltriflate was sufficient to produce [^{11}C]SB-235753 for its bioevaluation in rats further optimisation of radiochemical synthesis was non pursued.

Identity of the final radioactive product with 3-[^{11}C]methyl-(3-methoxy-naphthalen)-2-yl-(1-benzyl-piperidin-4-yl)-acetate was confirmed by co-injection with the authentic sample of SB-235753 on HPLC and by the mass spectrometric analysis of the product of the carrier-added synthesis (see experimental).The spectrum corresponded to that of (3-methoxy-naphthalen)-2-yl-(1-benzyl-piperidin-4-yl)-acetate. The large peak of non-radioactive material co-eluting with

[^{11}C]SB-235753 was observed on the u.v. trace of the preparative chromatogram. Identity of product was also confirmed by HPLC. The authentic sample of SB-235753 was co-injected with the labelled product. The product co-eluted with the standard.

Bioevaluation

Distribution of [^{11}C]SB-235753 was measured in rat brain at 5, 15, 30 min after intravenous injection of the tracer. Total injected mass of SB-235753 did not exceed 0.15 nmol per animal. Estimated density of D_4 receptors in rats is <30.0 fmol/mg protein (15).

The tracer moderately crossed the blood-brain barrier, the maximum uptake in the brain at 5 min being 0.35% of the injected dose. Fast wash-out of the radiotracer was observed from all studied brain areas and at 30 min 0.06% of the totally injected radioactivity remained in the brain. [^{11}C]SB-235753 was uniformly distributed within the brain suggesting non-specific uptake of the tracer or its metabolites (table 2).

Table 2. Regional brain distribution of [^{11}C]radioactivity in rats after injection of [^{11}C]SB-235753. (%I.D./g tissue; average of 3 rats ± SD).

Brain area	5 min	15 min	30 min
Striatum	0.1 ± 0.1	0.03 ± 0.03	0.01 ± 0.01
Anterior Cortex	0.08 ± 0.04	0.04 ± 0.03	0.02 ± 0.01
Posterior Cortex	0.07 ± 0.04	0.04 ± 0.04	0.02 ± 0.01
Striatum/Cerebellum	1.05 ± 0.25	0.93 ± 0.08	1.1 ± 0.1
Anterior Cortex/Cerebellum	0.9 ± 0.2	1 ± 0.2	1.1 ± 0.1
Posterior Cortex/Cerebellum	0.8 ±0.2	1 ± 0.2	1.3 ± 0.3
Plasma	0.4 ± 0.4	0.4 ± 0.3	0.2 ± 0.2

HPLC analysis indicated that polar metabolite(s) are present in plasma or rats injected with [^{11}C]SB-235753. In fact, retention time of the peak originated from the metabolite(s) was shorter (3 min) compared to that of the unmodified [^{11}C]SB-235753 (7.5 min).

Metabolic rate of the radiotracer was fast both in plasma and in the brain. Only 8% of the radioactivity found in plasma at 5 min corresponded to the unchanged [^{11}C]SB-235753 as assessed by HPLC (figure 1). At 30 min after injection 100% of the radioactivity in brain and 99% in the circulating blood was represented by one metabolic product(s) with retention time similar to that observed in the analysis of plasma extracts. Thus, due to the uniform distribution and rapid *in vivo* metabolism, rats studies suggested that [^{11}C]SB-235753 did not seem to be suitable for the *in vivo* evaluation of D_4 receptors.

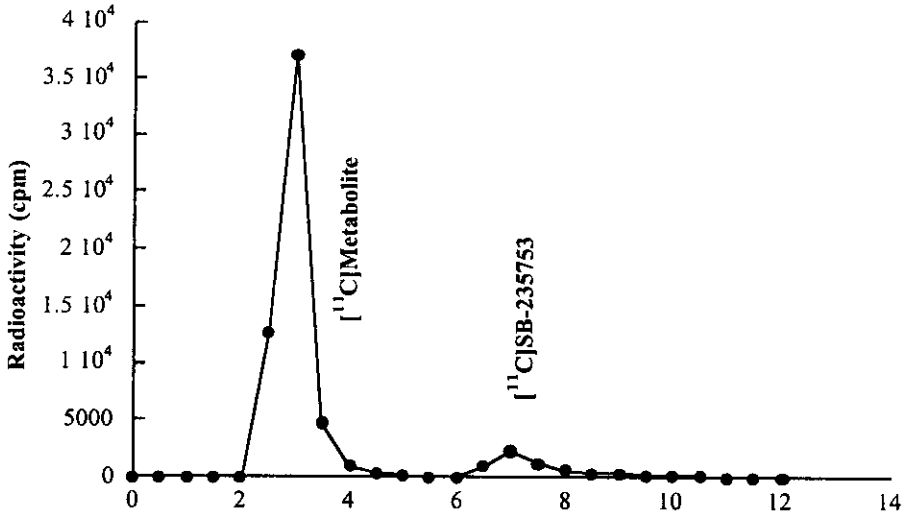


Figure 1. HPLC analysis of acetonitrile extract from blood sample collected at 5 min after injection of [^{11}C]SB-235753.

EXPERIMENTAL

Reagents and solvents were obtained from Aldrich Italia S.p.A. (Milano, Italy) and used without further purification unless otherwise noted. An authentic sample of SB-235753 (50 mg) was obtained from SmithKline & Beecham (UK).

[^{11}C]Carbon dioxide was produced by the ^{14}N(p,α)^{11}C reaction on a CTI-Siemens RDS-112 cyclotron, using 11.5 MeV proton beam at 10-30 μA currents. [^{11}C]Methyl iodide was synthesised by the standard one pot method (16), via lithium aluminium hydride reduction of [^{11}C]carbon dioxide, followed by hydriodic acid hydrolysis and distillation through an ascarite-sicapent purification column. [^{11}C]Methyl triflate was prepared as described by Jewett, 1992 (17) from [^{11}C]methyl iodide on a silver triflate-coated graphite column.

All radiochemical syntheses were performed on the fully automated synthesis module PET Tracer Synthesizer, Nuclear Interface Datentechnik GmbH, Münster, Germany. ^{1}H NMR spectra were recorded using a Bruker AC-200 spectrometer, in 0.05 M deuterochloroform solutions at 303 K; chemical shifts are reported as δ (ppm) relative to tetramethylsilane as external standard.

Mass spectra of SB-235753 and of intermediate products were recorded either by electron impact (EI-MS) or Fast Atom Bombardment (FAB-MS) ionization. In order to obtain information on the molecular mass of products spectrum by FAB-MS was recorded using a Finnigan MAT95 double-focusing mass spectrometer equipped with a caesium gun, recording positive ions and operating at an ion accelerating voltage of 5kV. Caesium ions were accelerated through 22kV. The spectra were recorded from m/z 50 or 100 to m/z 600 at 5 sec/decade. The instrument operated at a resolution of 1000 (10% valley). 3-Nitrobenzyl alcohol was used as the matrix for the analysis by FAB-MS of aliquots. The EI-MS spectra were obtained either by sample introduction into a particle beam HP 5988 instrument or by direct introduction into a Finnigan MAT95 and increasing the probe temperature at 5°C min^{-1} from 40°C up to 180°C. Both instruments were operating at 70 KeV.

GC was performed to measure the [^{11}C]methanol, [^{11}C]methyl iodide and [^{11}C]methyl triflate in the methylation mixture using a Carlo Erba gas chromatograph, equipped with TCD and flow radioactivity detector on Porapak Q column (1000 x 10 mm), 170°C, He 50 ml/min. TLC was performed on silica-coated glass plates (Merck, Silicagel 60 F_{254}).

The course of [^{11}C]methylation and quality control of the final product were accomplished by radio HPLC with acetonitrile - 0.05 M sodium dihydrogen orthophosphate (55:45, v/v, pH 7.3) at 2 ml/min, using Waters Millennium system equipped with u.v. detector set at 254 nm, and flow radioactivity detector. pH was measured on a Schott Geräte pH-meter. Sterility and pyrogenity tests were performed using standard procedures.

PRECURSOR SYNTHESIS

Ethyl-4-piperidylacetate (1).

Ethyl-4-pyridylacetate, (5 g, 30 mmol) was dissolved in 250 ml of water/methanol (1:2) followed by addition of Rhodium/Alumina catalyst (3.5 g). The reaction mixture was then hydrogenated at 3.5 atm for 20 hours in the stainless steel Parr apparatus. After filtration of catalyst and evaporation of solvents 5 g (29 mmol) of oil **1** was collected.

1-Benzylpiperid-4-yl-ethylacetate (2)

Ethyl-4-piperidylacetate **1** (500 mg, 2.92 mmol), titanium(IV)*iso*-propoxide (994 mg, 3.5 mmol) and benzaldehyde (310 mg, 2.92 mmol), were stirred under argon atmosphere for 1 hour followed by addition of absolute ethanol (2 ml) and sodium cyanoborohydride (183 mg, 2.92 mmol). The reaction mixture was left overnight, quenched with 1 ml of water and filtered. Purification by flash-chromatography on a silica column with dichloromethane/methanol mixture as an eluent (97:3) gave 580 mg of colourless oil **2** (76% yield, 98% purity by HPLC).

^{1}H-NMR ($CDCl_3$): δ 1.26 (t, 3H, CH_3), 1.37 (m, 2H, 2CH), 1.70 (m, 2H, 2CH), 1.80 (m, 1H, CH), 2.00 (m, 2H, 2CH), 2.24 (d, 2H, J = 7.0 Hz, $COCH_2$), 2.88 (m, 2H, 2CH), 3.50 (s, 2H, $PhCH_2$), 4.13(s, 2H, $PhCH_2$), 7.25-7.35 (m, 5H, Ph).
EI-MS: Molecular ion (m/z 261) and the base peak ion at m/z 91 (C_7H_7) with additional ions at m/z 232 (M-C_2H_5), at m/z 216 (M-C_2H_5O), at m/z 184 (M-C_6H_4), at m/z 170 (M-C_7H_6) and at m/z 188 (M-$C_3H_5O_2$).

1-Benzylpiperid-4-yl-acetic acid (3)

1-Benzylpiperid-4-yl-ethylacetate (450 mg, 1.72 mmol) was dissolved in 5 ml of ethanol followed by addition of 2 ml of 3 M sodium hydroxide. Hydrolysis was complete after 3 hours as monitored by TLC (dichloromethane/methanol 95:5). Upon evaporation of ethanol and addition of 20 ml of water the solution was acidified with acetic acid to pH 4.5 and washed with diethyl ether (2 x 10 ml). The organic layer was discarded. After saturation of the water solution with ammonium sulfate, the compound was extracted with dichloromethane (3 x 10 ml) and dried over sodium sulfate. Filtration and evaporation of solvent gave 400 mg (1.71 mmol, yield 99%) of yellow oil 3.
^{1}H-NMR ($CDCl_3$): δ 1.83 (m, 5H, 5CH), 2.28 (m, 2H, $COCH_2$), 2.54 (m, 2H, 2CH), 3.44 (m, 2H, 2CH), 4.13(s, 2H, $PhCH_2$), 4.13 (q, 2H, OCH_2), 7.30-7; 40 (m, 5H, Ph).
EI-MS: Molecular ion (m/z 233) and the base peak ion at m/z 232 (M-1) with additional ions at m/z 156 (M-C_6H_4), at m/z 142 (M-C_7H_6) and m/z 188 (M-$C_3H_5O_2$).

(3-Hydroxynaphthalen)-2-yl-(1-benzylpiperidin-4-yl)-acetate (4).

1-Benzylpiperid-4-yl-acetic acid (200 mg, 0.85 mmol) was dissolved in 7 ml of anhydrous dimethylformamide followed by addition of 2,3-dihydroxynaphtalin (140 mg, 0.87 mmol), and N-N-dicyclohexylcarbodiimide (175 mg, 0.85 mmol). After 1 hour stirring at room temperature, 200 mg of molecular sieves 4 Å were added to the reaction mixture and stirred overnight. Work-up was accomplished

by addition of 40 ml of water, extraction with ethyl acetate (3 x 25 ml) and after drying over magnesium sulphate and evaporating the solvent, the product was purified on a silica gel chromatography column. The non-reacted 2,3-dihydroxynaphtalin was washed out with diethyl ether/n-hexane (1:1; v/v). (3-Hydroxynaphthalen-2-yl)-(1-benzylpiperidin-4-yl)-acetate was eluted with diethyl ether to obtain 105 mg (33 % yield, 95% purity by HPLC) of white powder. The product was purified by crystallisation of its hydrochloride salt, 0.5 ml of 1 M HCl in diethyl ether was added to the base **4** dissolved in ethyl acetate to obtain 50 mg of hydrochloride salt of **4**, (99% purity by HPLC), which was used directly in the labelling procedures.

^{1}H-NMR ($DMSOd_6$): δ 1.66 (m, 2H, 2CH), 2.00 (m, 2H, 2CH), 2.08 (m, 1H, CH), 2.60 (d, 2H, J = 7.0 Hz, $COCH_2$), 2.98 (m, 2H, 2CH), 3.34 (m, 2H, 2CH), 4.26 (br s, 2H, $PhCH_2$), 7.20-7.80 (m, 11H, Ph), 10.10-10.40 (m, 2H, PhOH and NH^+).

EI-MS: Molecular ion (m/z 375) and the base peak ion at m/z 91 with two additional ions at m/z 216 and m/z 160 originated by the fragmentation of the molecule at the ester group.

RADIOSYNTHESIS

[^{11}C]SB-235753 (5). Into the closed reaction vial containing 1 mg (2.66 μmol) of precursor **4** and 10 μl (10 μmol) of 1 M aqueous solution of potassium hydroxide in dimethylformamide (100 μl) [^{11}C]methyl triflate was transported by a stream of argon (10 ml/min) at -15°C. At the end of trapping (3 min) the reaction mixture was diluted with 0.8 ml of mobile phase prior to injection into the HPLC semipreparative reversed phase column (Shandon Hypersil BDS C-18, 5 μm, 250 x 10 mm i.d.), eluted with acetonitrile-0.05M sodium dihydrogen orthophosphate (60/40; v/v; pH 7.4) at 4 ml/min. Retention times were confirmed before each synthesis by comparison with authentic standards and

corresponded to 8.15 min and 4.5 min for [^{11}C]SB-235753 and *des*-methyl precursor **4**, respectively. The effluent peak corresponding to [^{11}C]SB-235753 was collected in 40 ml of sterile water, and the product was recovered by solid phase extraction on pre-activated Sep-Pak C-18 cartridge (Millipore). The Sep-Pak was washed with water (10 ml) before eluting the product with ethanol (0.5 ml) into a vial containing 4.5 ml of saline solution. The final solution was sterilised through a sterile 0.22 μm filter (Gelman Acrodisc).

Determination of specific radioactivity, chemical and radiochemical purity.

The final solution of known volume was assayed for total radioactivity and a 20 μl aliquot was applied to an analytical column (Shandon Hypersil BDS C-18, 5 μm, 250 x 10 mm i.d.), eluted with a mobile phase of acetonitrile-0.05M sodium dihydrogen orthophosphate (55/45; v/v; pH 7.3) at 2 ml/min. Under these conditions, the retention time of [^{11}C]SB-235753 and *des*-methyl precursor **4** was 8 min, and 4 min, respectively. The amount of carrier was calculated from the u.v. absorbance peak area by means of the external standard calibration plot. The minimal detectable concentration was 0.2 nmole/ml. The radioligand was found to be radiochemically stable for at least 1 hour as assessed by HPLC, with a radiochemical purity over 97%.

In the typical experiment starting from 632 mCi of [^{11}C]CO_2, 76 mCi of the formulated [^{11}C]SB-235753 was obtained in 30 min in 10 ± 5% radiochemical yield with specific radioactivity of 10 ± 3 Ci/μmol and a chemical and radiochemical purity of 99%.

Carrier-added synthesis

The same synthetic procedure was used as described for the [^{11}C]SB-235753 synthesis, using following quantities: 1 mg (2.66 μmol) of *des*-methyl SB-235753, 10 μl (10 μmol) of 1 M aqueous solution of potassium hydroxide, 100 μl

of dimethylformamide; 10 µl of "cold" methyl iodide was added as a carrier to the hydriodic acid used for production of [^{11}C]methyl iodide. After collecting the fraction corresponding to [^{11}C]SB-235753 in 40 ml of sterile water, the product was recovered by solid phase extraction on preactivated Sep-Pak C-18 cartridge (Millipore) and eluted with 1 ml of methanol. After evaporation of methanol the product was analysed by EI MS.

Animal studies

Biodistribution studies in rats were performed ex-vivo in full conformity with the demands of the Laboratory Animal Ethical Committee (IACUC) of the Scientific Institute H S. Raffaele, Milan, Italy. Albino male CD rats aged 1-2 months and weighing 225-250 g, were obtained from Charles River (Italy).
Animals were injected in the tail vein with [^{11}C]SB-235753 in 300 µl of saline, injected dose was ranging from 200 to 700 µCi per animal with specific radioactivity 3 ± 1.5 Ci/µmol at the moment of injection. The biodistribution of [^{11}C]SB-235753 was assayed at 5, 15, 30 min (n =3 at each time point) after injection. Immediately after decapitation blood sample was collected into a heparinized tube, plasma and blood proteins was separated by centrifugation Regions of interest were cut off the brain, placed into pre-weighed tubes and assayed for the radioactivity by an automated well-counter (LKB Compugamma CS 1282). The uptake of radioactivity was calculated as a percentage of the injected dose per gram of tissue and corrected for decay (%I.D./g tissue).

The presence of radioactive metabolites of [^{11}C]SB-235753 in blood and brain tissue in rats were assessed by radio HPLC with acetonitrile - 0.05 M sodium dihydrogen orthophosphate (60:40; v/v) at 4 ml/min, on a Gilson 811C chromatograph equipped with autosampler and u.v. detector set at 254 nm. Blood samples were collected at 5 min (n=1), 15 min (n=2) and 30 min (n=2) after the injection of the tracer and immediately centrifuged at 3000g for 10 min in a Beckman microfuge. Plasma samples (0.5 ml) were extracted with 0.5 ml of

acetonitrile, the supernatant was filtered on a Millex-FG13 0.2 μm filter and injected into the reversed-phase column (Lichrosorb RP-18, 10 μm, 250 x 10mm). All radioactive fractions were collected and assayed for radioactivity on LKB-Compugamma 1282 automated gamma-counter.

Brain samples were obtained at 30 min (n=2). The tissue was homogenised in 2 ml of saline, and then extracted with acetonitrile. Radioactive metabolites were analysed by HPLC. An aliquot of cold SB-235753 was used as standard to verify the retention time of un-metabolised [^{11}C]SB-235753. The concentration of [^{11}C]SB-235753 and metabolites was expressed as percentages of activity in the acetonitrile extracts.

CONCLUSIONS

3-[^{11}C]Methyl-(3-methoxy-naphthalen)-2-yl-(1-Benzyl-piperidin)-4-yl-Acetate (SB-235753), was prepared by *O*-methylation of the *des*-methyl precursor in sufficient quantity and with high specific radioactivity for studies by positron emission tomography. Biological evaluation in rats has shown that [^{11}C]SB-235753 is not a suitable radioligand for dopamine D_4 receptor studies with PET, due its rapid metabolic degradation. Whether the tracer metabolism is species specific need further evaluation in other animal models.

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