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Labelling and biological evaluation of [^{11}C]methoxy-Sch225336: a radioligand for the cannabinoid-type 2 receptor

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Abstract

Introduction: The cannabinoid type 2 receptor (CB_2 receptor) is part of the endocannabinoid system and has been suggested as mediator of a number of central and peripheral inflammatory processes. In the present study, we have synthesized *N*-[(1s)-1-[4-[[4-methoxy-2-[(4-[^{11}C]methoxyphenyl)sulfonyl)-phenyl]sulfonyl] phenyl]ethyl]methanesulfonamide ([^{11}C]methoxy-Sch225336) and evaluated this new tracer agent as a potential positron emission tomography radioligand for the in vivo visualization of CB_2 receptors.
Methods: Sch225336 was demethylated and the resulting phenol precursor was radiolabelled with a carbon-11 methyl group by methylation using [^{11}C]methyl iodide, followed by purification by high-performance liquid chromatography. The log P of [^{11}C]methoxy-Sch225336 and its biodistribution in normal mice were determined. Enhancement of brain uptake by inhibition of blood-brain barrier (BBB) efflux transporters was studied. Mouse plasma was analysed to quantify the formation of radiometabolites. The affinity of Sch225336 for the human cannabinoid type 1 and type 2 receptor was determined.
Results: [^{11}C]methoxy-Sch225336 was obtained with a decay corrected radiochemical yield of about 30% and a specific activity of 88.8 GBq/μmol (end of synthesis). After intravenous injection in mice, the compound is rapidly cleared from the blood through the hepatobiliary pathway and does not show particular retention in any of the major organs. Polar metabolites were found in mouse plasma. Brain uptake was low despite the favourable log *P* value of 2.15, which is partly due to efflux by BBB pumps.
Conclusion: [^{11}C]methoxy-Sch225336 is a good candidate for in vivo imaging of the CB_2 receptor, although the low blood-brain barrier penetration limits its potential for central nervous system imaging.

Keywords: Cannabinoids; CB_2 receptor; PET; Carbon-11; Sch225336

1. Introduction

Two seven-transmembrane G protein-coupled cannabinoid receptors have been identified and molecularly characterized so far, namely, the cannabinoid type 1 receptor (CB_1) and type 2 receptor (CB_2) [1,2]. Pharmacological experiments have tentatively indicated the presence of other cannabinoid receptors, but these have not yet been cloned [3].

CB_1 and CB_2 receptors are part of the endocannabinoid system which also comprises, among others, the endogenous ligands anandamide and 2-arachidonoyl glycerol and the catabolic enzymes fatty acid amide hydrolase and monoglyceride lipase.

While CB_1 is one of the most abundant neuromodulatory receptors in the brain, both CB_1 and CB_2 receptors are widely distributed in peripheral tissues, with CB_2 receptors particularly enriched in immune tissues. The presence of

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the CB_2 receptor has been reported in spleen, lymph nodes, Peyer's patches, blood and bone marrow, whereas thymus, liver and lung are devoid of CB_2 receptors [4]. In the central nervous system (CNS), CB_2 receptors appear in low concentration in the cerebellum, hippocampal pyramidal cells and brain stem [5–7], as well as on activated microglia [8]. CB_2 receptor up-regulation has been reported in various pathological conditions such as human astrocytic tumours where the level of CB_2 receptor expression correlates with tumour malignancy [9]. Expression of the CB_2 receptor was also demonstrated in human and mouse atherosclerotic plaques, senile amyloid plaques in Alzheimer patients and plaques of demyelination in multiple sclerosis patients [10–12].

The CB_2 receptor has been suggested as a valuable clinical target for treating atherosclerosis and suppressing certain symptoms of multiple sclerosis [10,13]. Several CB_2 receptor agonists have been synthesized and were found to have positive effects in various forms of inflammation and inflammatory or neuropathic pain, and they were reported to induce apoptosis in different tumour cells [14–17].

At this moment, a number of radioligands with affinity for the human CB_2 receptor for in vitro use are available. Nonselective cannabinoid radioligands such as [^{3}H]CP55,940 or [^{3}H]WIN55,212 are extensively used in binding analyses of the CB_2 receptor. Although these ligands bind with low nanomolar affinity, they have poor specific activities inherent to tritiated radioligands. Recently, Gonsiorek et al. described a new radioligand, [^{35}S]Sch225336, which has a subnanomolar affinity for the human CB_2 receptor (hCB_2 receptor) and was obtained with a high specific activity (>52 GBq/μmol) [18]. Therefore, this compound can be very useful for in vitro studies of endogenously expressed CB_2 receptors. In addition, synthesis of a near-infrared dye labelled CB_2 receptor ligand for noninvasive in vivo imaging was reported recently [19].

To our knowledge, no radioligands for positron emission tomography (PET) or single photon emission computed tomography have been described so far for in vivo visualization of the CB_2 receptor. In the present study, we have synthesized and studied the biological behaviour of carbon-11 labelled *N*-[(1s)-1-[4-[[4-methoxy-2-[(4-methoxyphenyl)sulfonyl)-phenyl]sulfonyl] phenyl]ethyl]methanesulfonamide (Sch225336), a radiolabelled analog of a novel CB_2-selective triaryl *bis*-sulfone [20,21], as a potential CB_2 receptor radiotracer agent for PET. Sch225336 is an inverse agonist with high affinity for the CB_2 receptor (K_i hCB_2=0.4 nM) and has a good selectivity relative to the CB_1 receptor (K_i hCB_1=905 nM) [20].

2. Methods

2.1. Chemicals and reagents

The starting compound for this study was Sch225336, which was kindly provided by the Schering Plough Research Institute, Kenilworth, NJ, USA. All reagents and solvents were obtained commercially from Aldrich (Steinheim, Germany) or Acros (Geel, Belgium) and were used as such without further purification. Cyclosporin A (CsA; Sandimmun) was purchased from Novartis Pharma (Vilvoorde, Belgium).

2.2. Apparatus, instruments and general conditions

All glassware was dried in an oven at 110°C for several hours and allowed to cool to room temperature (RT) before use. For ascending thin layer chromatography, precoated aluminium backed plates (Silica gel 60 with fluorescence indicator, 0.2 mm thickness; supplied by Macherey-Nagel, Düren, Germany) were used and developed using 4% MeOH in CH_2Cl_2 as mobile phase. After evaporation of the solvent, compounds were detected under UV light (254 nm). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance II 500 spectrometer (Fällanden, Switzerland) operating at 500.130 MHz for ^{1}H, or at 125.758 MHz for ^{13}C, and using a gradient equipped inverse 5-mm triple probe with π/2 pulses of 6.5 and 14.5 μs, respectively. The standard Bruker Topspin 1.3 software under Windows XP was used. All experiments were performed at 22°C in deuterated chloroform. Chemical shifts are reported in parts per million relative to trimethylsilane (δ=0) or relative to the solvent peak as internal standard set at 77.0 ppm (^{13}C) vs. tetramethylsilane. First-order analysis was applied throughout, and first-order multiplets or apparent first-order multiplets are denoted as follows: s=singlet, d=doublet, dd=double doublet, t=triplet, q=quadruplet, m=multiplet, obs/ovl=obscured or overlapped signal. *J* values were extracted directly from the splittings in the spectrum and are not optimised. Spectral assignments were based not only on the usual chemical shift rules and coupling patterns but especially on routine 2D correlations such as COSY45 (homonuclear H,H J-correlations), GHSQC (single bond C,H ^{1}J-correlations) and GHMBC experiments (multiple bond C,H ^{3}J-correlations). High-performance liquid chromatography (HPLC) analysis was performed using an XTerra RP18 column (5 μm, 4.6 mm×250 mm; Waters, Milford, MA, USA) eluted with 40 % CH_3CN in 0.05 M ammonium acetate buffer (pH 6.8) at a flow rate of 1 ml/min. The column effluent was monitored using an UV detector set at 254 nm and the output signal was recorded and analyzed using the RaChel analysis program (Lablogic, Sheffield, UK). The purity of the nonradioactive compounds determined using the above described HPLC method was found to be >98%. For analysis of radiolabelled compounds, the HPLC eluate was led over a 3-inch NaI(Tl) scintillation detector connected to a single channel analyzer (Medi-Lab Select, Mechelen, Belgium). The radioactivity measurements during biodistribution studies and blood metabolite studies were done using a gamma counter [3-in. NaI(Tl) well crystal] coupled to a multichannel analyzer and mounted in a sample changer (Wallac 1480 Wizard 3", Wallac, Turku,

Finland). The values are corrected for background radiation and physical decay during counting. Exact mass measurement was performed on a time-of-flight mass spectrometer (LCT, Micromass, Manchester, UK) equipped with an orthogonal electrospray ionization interface, operated in positive mode (ES+). Samples were infused in CH_3CN/water using a Harvard 22 syringe pump (Harvard instruments, Holliston, MA, USA). Accurate mass determination was done by coinfusion with a 100 μg/ml solution of 3-(2′-deoxy-β-D-ribofuranosyl)-6-(3-hydroxy, 4-iodophenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one as an internal lock mass. Acquisition and processing of data was done using Masslynx software (version 3.5).

2.3. Synthesis

2.3.1. *N-[(1s)-1-[4-[[4-methoxy-2-[(4-hydroxyphenyl)sulfonyl]-phenyl]sulfonyl]phenyl]ethyl]methanesulfonamide* **(2)**

To a stirred solution of Sch225336 (0.100 g, 0.185 mmol) in dry dichloromethane under nitrogen at −70°C was added a 1 M solution of boron tribromide in CH_2Cl_2 (5 eq, 0.927 ml) dropwise over 30–45 min. The mixture was maintained at −70°C for 1 h and then allowed to warm slowly to RT at which it was stirred overnight. The reaction mixture was again cooled to −70°C and the excess of boron tribromide was quenched by dropwise addition of methanol until no further reaction occurred. The reaction mixture {containing two mono-methoxy derivatives **(1)** and **(2)**} was purified by column chromatography on silica gel (63-200-μm particle size, 60 Å, MPBiomedicals, Eschwege, Germany) using dichloromethane-methanol (99:1) as the eluent. The purified fractions (40 mg), however, contained mixtures of both mono-methoxy derivatives **(1)** and **(2)** that were further separated using semipreparative reversed-phase HPLC (RP-HPLC) on an XTerra RP18 column (10 μm, 10 mm×250 mm, Waters) eluted with ammonium acetate 0.05 M/acetonitrile (60:40 V/V) at a flow rate of 2.6 ml/min. The mixture (10 mg) was separated by repeated injection of fractions of 500 μg dissolved in 250 μl acetonitrile. The combined fractions containing pure isomer **(2)** were passed over an activated SepPak plus C18 cartridge (Waters), which was then rinsed with water (3 ml). The cartridge was dried with a stream of nitrogen for 15 min followed by elution with 5 ml dichloromethane. The resulting solution was evaporated to yield 5.4 mg of pure isomer **(2)** (overall yield 20%). The structure of isomer **(2)** was confirmed by high-resolution mass spectrometry (MS), ^{1}H NMR and ^{13}C NMR. MS $(ES)^+$ accurate mass: $[C_{22}H_{22}NO_8S_3 + Na]^+$ theoretical mass 548.0478 Da and found 548.0516 Da. ^{1}H NMR ($CDCl_3$) δ 8.452 (1H, d, $^3J_{H\text{-}H}$=8.5 Hz, *12*), 7.942 (1H, d, $^4J_{H\text{-}H}$=3.0 Hz, *9*), 7.803 (2H, d, $^3J_{H\text{-}H}$=8.5 Hz, *3* and *5*), 7.786 (2H, d, $^3J_{H\text{-}H}$=8.5 Hz, *14* and *18*), 7.434 (2H, d, $^3J_{H\text{-}H}$=8.5 Hz, *2* and *6*), 7.228 (1H, dd, $^3J_{H\text{-}H}$=8.7, $^4J_{H\text{-}H}$=2.8, *11*), 6.846 (2H, d, $^3J_{H\text{-}H}$=8.5, *15* and *17*), 4.871 (1H, d, $^3J_{H\text{-}H}$=7.0, *NH*) 4.697 (1H, m, *20*), 3.972 (3H, s, *19*), 2.724 (3H, s, *22*), 1.519 (3H, d, $^3J_{H\text{-}H}$=7.0, *21*) ppm.

^{13}C NMR ($CDCl_3$) 163.68 (C*10*), 160.67 (C*16*), 147.56 (C*1*), 142.92 (C*8*), 141.71 (C*4*), 135.88 (C*12*), 131.68 (C*13*), 130.82 (C*14* and C*18*), 129.75 (C*7*), 127.97 (C*3* and C*5*), 126.49 (C*2* and C*6*), 119.49 (C*9*), 117.00 (C*11*), 115.61 (C*15* and C*17*), 56.33 (C*19*), 53.16 (C*20*), 42.03 (C*22*), 23.75 (C*21*) ppm.

2.4. Radiolabelling and HPLC

Carbon-11 was produced in a Cyclone 18/9 cyclotron (IBA, Louvain-la-Neuve, Belgium) in the form of $^{11}CH_4$ by irradiation of a mixture of N_2 with 5 % H_2 with 18-MeV protons. $^{11}CH_4$ was transferred from the cyclotron target to a homemade synthesis module housed in a lead-shielded cabinet where $^{11}CH_4$ was trapped on a column filled with Porapak N (divinylbenzene/vinyl pyrolidone polymer), cooled in liquid nitrogen. The loop was allowed to warm to RT. $^{11}CH_4$ was swept off the column with a stream of helium and was mixed with I_2-vapour at 100°C, after which the mixture passed through a quartz column heated to 650°C to yield $^{11}CH_3I$ [22]. $^{11}CH_3I$ was used as such or passed through a column (150×3 mm) filled with silver triflate (50 mm silver triflate between two times 50 mm quartz wool) heated to 180°C, yielding the more reactive [^{11}C]methyl triflate. A stream of helium containing the alkylating agent was bubbled through a solution of 200-μg precursor **(2)** and 2-4 mg Cs_2CO_3 in 200 μl dimethylformamide (DMF). The reaction mixture was heated during 4 min at 90°C and then diluted with 1.8 ml 0.05 M ammonium acetate buffer pH 6.9 and applied onto a semi-preparative XTerra RP18 column (5 μm, 7.8×150 mm; Waters) which was eluted with 0.05 M ammonium acetate buffer (pH=6.9)/EtOH (45:55 V/V) at a flow rate of 2 ml/min. For biodistribution studies, the isolated peak containing the radiolabelled compound was diluted at least tenfold using saline.

Quality control was performed using HPLC on an XTerra RP18 column (5 μm, 4.6×250 mm, Waters) eluted with 0.05 M ammonium acetate buffer pH 6.9/acetonitrile (50:50 V/V) at a flow rate of 1 ml/min.

2.5. Cell culture

All cell culture media and supplements were obtained from Invitrogen (Merelbeke, Belgium). Transfected CHO cells stably expressing the human CB_1 or CB_2 receptors (CHO CB_1 and CHO CB_2), kindly provided by Euroscreen (Gosselies, Belgium) were maintained using Ham's F12 medium supplemented with 10% foetal calf serum, 500 μg/ml geneticin G418, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone/amphotericin B and 2 mM L-glutamine. At confluence, cells were trypsinized for dilutions. Cells were cultured at 37°C in an atmosphere of humidified air and 5% CO_2.

2.6. Membrane preparation

Cells were lysed in ice-cold homogenization buffer containing 50 mM Tris-HCl, pH 7.4. The homogenate was

centrifuged at 15000×*g* for 10 min. The resulting membrane pellet was washed twice under the same conditions, resuspended in the same buffer and frozen (−80°C) in aliquots until use. Protein content was determined using the Bradford method with bovine serum albumin as standard [23].

2.7. Competition binding assay

The assay was performed as previously described [24]. Briefly, competition experiments were conducted on 40 μg CHO-CB_1 or CHO-CB_2 membrane preparation incubated with 1 nM [^{3}H]-SR141716A (Amersham, Roosendaal, the Netherlands) or [^{3}H]-CP55,940 (NEN Life Sciences, Zaventem, Belgium) for determination of the affinity for hCB_1R and hCB_2R, respectively, and with decreasing concentrations of various competition ligands, in plastic tubes containing 0.5 ml final volume of binding buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.5% bovine serum albumin, pH 7.4). Nonspecific binding was determined in the presence of 10 μM HU 210, a nonselective CB_1R and CB_2R agonist (Tocris Bioscience, Bristol, UK). After incubation for 1 h at 30°C, the solutions were filtered through 0.5% polyethyleneimine pretreated glass fiber filters (Whatman, Maidstone, UK).

Under these conditions, using [^{3}H]-SR141716A the B_{max} value was 57 pmol/mg of protein and the K_d value was 1.13±0.13 nM for the hCB_1 receptor. For the hCB_2 receptor B_{max} and K_d values were 194.2 pmol/mg of protein and 4.3±0.13 nM, respectively.

2.8. [^{35}S]-GTPγS Assay

A [^{35}S]-GTPγS assay was performed for evaluation of the compounds' potency and efficacy in signal transduction. Experiments were carried out, as previously described, in a 0.5-ml total volume of buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 0.1% bovine serum albumin, pH 7.4) containing 20 μM GDP, 40 μg protein samples from membrane preparation, decreasing concentrations of the tested compound and 0.05 nM [^{35}S]-GTPγS (Amersham, Roosendaal, the Netherlands) [24]. Tubes were incubated for 1 h at 30°C before filtration through nonpretreated glass fiber filters. Nonspecific binding was determined using 100 μM Gpp(NH)p.

2.9. Data analysis

IC_{50}, EC_{50} and efficacy were determined from at least three separate experiments by nonlinear regression analysis performed using Graph Pad Prism software (San Diego, CA). K_i values were calculated following the Cheng-Prusoff equation: $K_i = IC_{50}/(1+L/K_d)$ where L is the radioligand concentration [25].

2.10. Biodistribution studies

The experiments in mice were carried out in compliance with the national laws relating to the conduct of animal experimentation and approved by the local Animal Ethics Committee. Animals had free access to water and food during the experimental period. The biodistribution of *N*-[(1s)-1-[4-[[4-methoxy-2-[(4-[^{11}C]methoxyphenyl)sulfonyl)-phenyl]sulfonyl] phenyl]ethyl]methanesulfonamide ([^{11}C]methoxy-Sch225336) was studied in male National Murine Research Institute (NMRI) mice (body mass 34-41 g) at 2 min and 60 min post injection (n=4 at each time point). The mice were anesthetized by use of isoflurane (2% in oxygen). The solution of the HPLC-purified product was diluted with saline to a concentration of approximately 90 MBq/ml. An aliquot of 100 μl was injected via a tail vein. The mice were sacrificed by decapitation and the organs and body parts were dissected and weighed. The activity in the dissected organs and blood was measured using an automatic gamma counter coupled to a multi-channel analyzer. Results are expressed as percentage of injected dose (% of ID) and percentage of injected dose per gram tissue (% of ID/g). For calculation of total blood radioactivity, blood mass was assumed to be 7 % of the body mass.

2.11. Blood-brain barrier transporter inhibition study

The biodistribution of [^{11}C]methoxy-Sch225336 was studied in male NMRI mice (body mass 35–42 g) at 2 min post injection in the presence of CsA or vehicle (n=4 or n=3 at each time point). The mice were anesthetized by use of isoflurane. One group of mice was pretreated with CsA (50 mg/kg, 200 μl) by intravenous injection. The second group of mice was a control group and was pretreated with an intravenous injection of 200 μl of the injection vehicle of CsA (each ml vehicle contains 130 mg cremophor EL and 6.6% ethanol in NaCl 0.9%). After 30 min, an aliquot of [^{11}C]methoxy-Sch225336 (~9.25 MBq in 100 μl 0.9 % NaCl) was injected via a tail vein. The mice were sacrificed after 2 min by decapitation, and the organs and body parts were dissected and weighed. The activity in the dissected organs and blood was measured using an automatic gamma counter coupled to a multichannel analyzer. Results are expressed as % of ID and % of ID/g. For calculation of total blood radioactivity, blood mass was assumed to be 7% of the body mass. All statistical analyses were performed with the unpaired two-sided Student's t test. A P value of less than 0.01 was considered statistically significant.

2.12. Partition coefficient

25 μl of a solution of the HPLC isolated [^{11}C]methoxy-Sch225336 was added to a test tube containing 2 ml of 1-octanol and 2 ml of 0.025 M phosphate buffer, pH 7.4. The test tube was vortexed at RT for 2 min and then centrifuged at 2700 g for 10 min. A 100-μl aliquot was taken from the 1-octanol phase and a 900-μl aliquot from the aqueous phase, taking care to avoid cross contamination between the phases. The separate aliquots were transferred into tared vials, and the volume added was calculated from the mass of the aliquots and the specific density (ρ) of the phase,

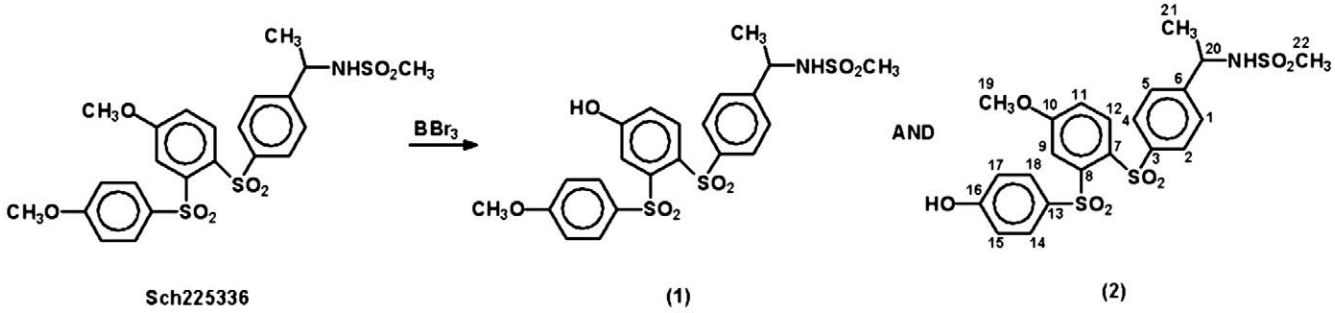

Fig. 1. Demethylation of Sch225336 resulting in the formation of two mono-methoxy isomers.

assuming that ρ_{buffer}=1.000 g/ml and $\rho_{1\text{-octanol}}$=0.827 g/ml. The radioactivity of the aliquots was counted using an automatic γ-counter. The partition coefficient (*P*) was calculated as [radioactivity (cpm/ml) in 1-octanol]/[radioactivity (cpm/ml) in phosphate buffer pH 7.4].

2.13. Blood metabolites

The in vivo metabolic stability of [^{11}C]methoxy-Sch225336 in blood was studied in male NMRI mice. An activity of 9.25 MBq of [^{11}C]methoxy-Sch225336 was injected through a tail vein, and the mice were sacrificed by decapitation at 2, 10 or 30 min post injection (p.i.) (one mouse per time point). Blood was collected into a BD vacutainer (containing 7.2 mg K_2EDTA; Beckton Dickinson, Franklin Lakes, USA). The samples were then centrifuged at 3000 rpm (1837 g) for 5 min to separate plasma. The supernatant plasma was mixed with 10 µl of authentic Sch225336 (1 mg/ml) and injected onto an Oasis HLB column (Hydrophilic-Lipophilic Balanced; 4.6×20 mm, Waters) that was preconditioned by successive washings with acetonitrile and water [26]. The proteins of the plasma matrix were washed from the Oasis column with 10 ml of water, which was collected as two 5-ml fractions (fraction 1 and 2). The outlet of the Oasis column was then connected to an analytical XTerra RP18 HPLC column (5 µm, 4.6×250 mm; Waters), and both columns in series were then eluted using 0.05 M ammonium acetate/acetonitrile (40/60 V/V) as the mobile phase at a flow rate of 1 ml/min. The HPLC-eluate was collected in 1-ml fractions and their radioactivity as well as the activity in fractions 1 and 2 was measured using an automatic gamma counter.

3. Results

3.1. Synthesis and radiolabelling

Sch225336 (Fig. 1) is a bismethoxy triaryl *bis*-sulfone of which, in the present study, the [^{11}C]methoxy analog was synthesized as a radioligand for PET. Conversion of Sch225336 into a mono-methoxy derivative was performed using a demethylation procedure with BBr_3 at −70°C, as described by McOmie et al. [27]. A mixture of two mono-methoxy derivatives [**(1)** and **(2)**] was obtained (Fig. 1), as indicated by HPLC and MS analysis. The crude reaction mixture was first purified using silicagel column chromatography and the isomers were separated by preparative RP-HPLC. Monomethoxy derivatives **(1)** and **(2)** were obtained in a relative ratio of 25:75. The structure of **(2)** was confirmed using ^{1}H NMR and ^{13}C NMR. Overall reaction yield of compound **(2)** was 2.8%. This low yield was due to the instability of **(2)** during the purification process. Compound **(2)** was used as precursor for radiochemical synthesis of [^{11}C]methoxy-Sch225336.

A carbon-11 methylation yield of about 35 % was obtained by heating a solution of 200 µg **(2)** in 200 µl DMF in the presence of [^{11}C]CH_3I and Cs_2CO_3 during 4 min at

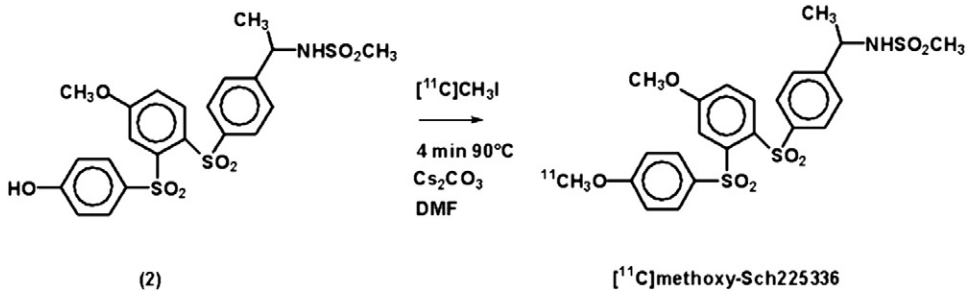

Fig. 2. Radiosynthesis of [^{11}C]methoxy-Sch225336 using [^{11}C]CH_3I.

90°C. Similar labelling yields were obtained using [^{11}C] methyl triflate as alkylating agent, whereas alkylation (with [^{11}C]CH_3I) at RT gave low radiochemical yields (~4% after 5 min) (Fig. 2).

The radiolabelled reaction product was separated from the precursor using RP-HPLC yielding >99% pure [^{11}C] methoxy-Sch225336 with a specific activity of 88.8 GBq/μmol (average of five labellings; at end of synthesis). The identity of the tracer was confirmed by coelution with authentic reference compound Sch225336 after coinjection on HPLC (Fig. 3).

3.2. Partition coefficient

The lipophilicity of [^{11}C]methoxy-Sch225336 was determined by partitioning between 1-octanol and 0.025 M phosphate buffer pH 7.4. The log *P* of [^{11}C]methoxy-Sch225336 was 2.15.

3.3. Competition binding studies and [^{35}S]-GTPγS assay

The affinity of Sch225336 for the human CB_1 and CB_2 receptor (hCB_1R and hCB_2R) was assayed using a competition binding assay on hCB_1R or hCB_2R transfected CHO cells with [^{3}H]-SR141716A or [^{3}H]-CP55,940 as radioligand. The affinity of Sch225336 was found to be 78.5±10.4 nM for hCB_1R and 4.54±0.48 nM for hCB_2R. The [^{35}S]-GTPγS assay indicated that Sch225336 acts as an inverse agonist on the hCB_2 receptor.

3.4. Biodistribution and plasma metabolite studies

Biodistribution of [^{11}C]methoxy-Sch225336 was studied in normal NMRI mice (Table 1). At 60 min p.i. 11% of ID was found in the liver, and 71% of ID, in the intestines. A smaller amount of [^{11}C]methoxy-Sch225336 was found in the kidneys (8.0% of ID and 1.1% of ID at 2 and 60 min p.i., respectively), but almost no activity was excreted into urine. A high initial uptake was observed in liver, lungs and heart, but except for the liver, the studied organs do not show retention of [^{11}C]methoxy-Sch225336. Brain uptake of [^{11}C] methoxy-Sch225336 was very limited (Table 1). The activity found in blood dropped from 4.1% of ID at 2 min p.i. to 1.2% of ID at 60 min p.i.

Plasma obtained from mice at 2, 10 and 30 min p.i. was analysed by RP-HPLC to determine the presence and relative amounts of radiometabolites of [^{11}C]methoxy-Sch225336. The relative percentage of intact [^{11}C]-Sch225336 as a function of time after injection was 93% at 2 min p.i., 63% at 10 min p.i. and 60 % at 30 min p.i. All metabolites detected were more polar than [^{11}C] methoxy-Sch225336).

3.5. Blood-brain barrier transporter inhibition study

Pretreatment of mice with cyclosporin A resulted in a significantly higher brain uptake (Table 2) compared to control, also when cerebrum and cerebellum were taken into account separately. Blood values of [^{11}C]methoxy-Sch225336 were twice as high in the CsA treatment group compared to the control group. However, brain-to-blood ratio was also significantly different between both groups. The biodistribution results of the vehicle pretreated group were not significantly different from the results shown in Table 1 (data not shown), except for uptake in cerebrum (0.07±0.00 for nonpretreated against 0.05±0.01 for vehicle pretreated group). Nevertheless, total brain uptake (cerebrum +cerebellum) was not significantly different.

Table 1
Biodistribution of [^{11}C]methoxy-Sch225336 at 2 and 60 min post injection in mice (n=4 at each time point) (SD=standard deviation)

Organ	% of ID±S.D.[a]		% of ID/g±S.D.	
	2 min	60 min	2 min	60 min
Kidneys	8.0±1.0	1.1±0.3	10.9±1.9	1.6±0.2
Urine	0.1±0.0	0.3±0.1		
Liver	51.5±9.1	10.8±1.7	24.4±2.7	4.6±0.7
Spleen	0.5±0.2	0.1±0.0	4.1±1.3	0.6±0.1
Pancreas	0.9±0.2	0.3±0.1	4.1±0.6	1.2±0.1
Lungs	1.5±0.3	0.4±0.1	4.5±0.8	1.1±0.2
Heart	1.1±0.2	0.1±0.0	5.9±1.4	0.6±0.0
Intestines	12.6±3.0	71.2±6.9		
Stomach	1.8±0.5	0.5±0.2		
Blood	4.1±1.2	1.2±0.1	1.6±0.4	0.4±0.0
Brain	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.1

[a] Percentage of injected dose calculated as cpm in organ/ total cpm recovered.

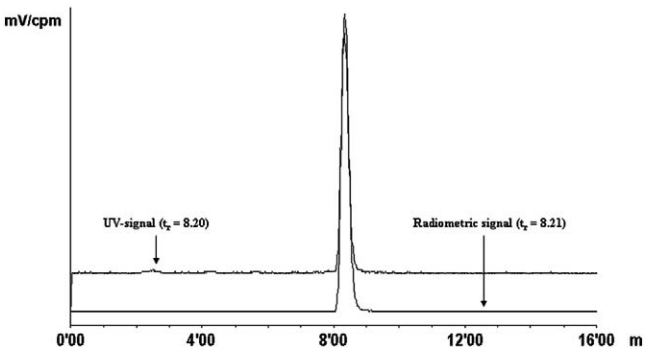

Fig. 3. HPLC analysis of [^{11}C]methoxy-Sch225336 after coinjection with reference compound Sch225336.

Table 2
Biodistribution of [^{11}C]methoxy-Sch225336 at 2 min post injection in the presence of cyclosporin A (treatment) or vehicle (control) in mice

	Control (% ID±S.D.)	Treatment (% ID±S.D.)
Cerebrum	0.05±0.01	0.16±0.02*
Cerebellum	0.01±0.01	0.08±0.01*
Total brain	0.07±0.01	0.24±0.02*
Blood	5.57±0.49	11.56±1.61*
Brain to blood ratio	0.01±0.00	0.02±0.00*

Data are mean±S.D. (n=3–4).
* $P<0.01$ (Student's t test, compared with control).

4. Discussion and conclusion

Although [^{11}C]methoxy-Sch225336 has favourable physicochemical characteristics [molecular weight <600 Da, not charged, log P 2.15] to pass the blood-brain barrier (BBB) [28], [^{11}C]methoxy-Sch225336 showed very limited brain uptake. This implicates that in vivo pharmacologic tests with Sch225336 will be devoid of central effects, but the very low brain uptake will also limit the potential use of a radiolabelled analog to a tracer agent for visualization of peripheral CB_2 receptor expression only. As mentioned in the introduction, such tracer agent could be a powerful tool to study the role of CB_2 receptors in vivo in several pathological conditions such as peripheral inflammation or atherosclerotic plaques.

Lipophilic compounds with low molecular weight like Sch225336 are normally entering the brain by passive diffusion. However, [^{11}C]methoxy-Sch225336 showed negligible brain uptake. In literature, other compounds containing sulfonyl groups also showed poor BBB penetration [29]. If these compounds act as a substrate for transporters as P-glycoprotein, multidrug resistance-associated protein or organic anion transporting polypeptides, this could partly explain the lack in brain uptake. In this study, we observed a significant difference in brain uptake of [^{11}C]methoxy-Sch225336 in mice in the presence of CsA as compared to a control group. Pretreatment of NMRI mice with CsA caused an almost fourfold increase of the brain uptake of [^{11}C]methoxy-Sch225336 and a twofold increase of residual blood activity. So, the increased brain uptake after pretreatment with CsA is probably partially due to modulation of transporters in the BBB and, partially, to reduced clearance of the radioligand. This means the absence of tracer uptake or retention in brain can at least partly be ascribed to efflux by transporter proteins. Sch225336 also has a large polar surface area (theoretical PSA=132 Å^2), which is defined as the sum of surfaces of polar atoms in a molecule [30]. Waterbeemd et al. [31] showed that, of a set of 125 drugs, all those showing CNS activity could be found within the ranges $0 \leq PSA \leq 90$, with the likelihood of CNS activity appearing to increase with decreasing PSA. Sch225336 probably also has a high hydrogen bound interaction which in addition will lower brain uptake.

[^{11}C]methoxy-Sch225336 is actively metabolized, so some of the metabolites may have affinity for BBB transporters. However, the percentage of metabolites in plasma is still small 2 min p.i., and all metabolites of [^{11}C]methoxy-Sch225336 are more hydrophilic than the parent compound, so they can be expected to show negligible brain uptake.

The results of the biodistribution studies in normal mice indicate that the compound is cleared from plasma mainly through the hepatobiliary pathway as the decrease in liver uptake parallels the increase in intestinal uptake. It thus will be impossible to discernate specific binding to the CB_2 receptor expressed in the intestines (Peyer's patches) from the presence of activity in the intestines due to hepatobiliary clearance of the compound or its radiometabolites [4]. A relatively high tracer concentration was observed in the kidneys at 2 min p.i., but only a very low activity was found in urine 60 min p.i., indicating that there is no important excretion of [^{11}C]methoxy-Sch225336 through the kidneys. Despite the reported high CB_2 receptor expression in the spleen, no high uptake or retention of [^{11}C]methoxy-Sch225336 in spleen was observed [32]. Plasma metabolite studies demonstrated important metabolism of [^{11}C]methoxy-Sch225336 with generation of more polar metabolites. The metabolites have not been identified nor have we determined their affinity for the CB_2 receptor.

The K_i values obtained in the competition binding studies ($K_i hCB_1$=78.5 nM and $K_i hCB_2$=4.5 nM) are somewhat different (especially regarding the selectivity for CB_2R relative to CB_1R) from the values reported in literature ($K_i hCB_1$=905 nM and $K_i hCB_2$=0.4 nM) [20,33]. This could be due to a difference in experimental conditions (1-h incubation in our assay instead of 2-h incubation), but more likely, the difference is due to the use of another radioligand for the assay ([^{3}H]-SR141716A instead of [^{3}H]-CP 55,940) [34]. [^{3}H]-SR141716A is a selective CB_1 inverse agonist, while [^{3}H]-CP55,940 is a nonselective CB_1 and CB_2 receptor agonist.

In a [^{35}S]-GTPγS assay, both Sch225336 and SR144528, which is frequently used as general CB_2 receptor selective inverse agonist, display a comparable potency (EC_{50}=9.91±5.5 nM and 2.1±1.1 nM respectively). However, Sch225336 seems to possess a better negative efficacy (E_{max}: −75% relative to basal specific [^{35}S]-GTPγS binding) than SR144528 (E_{max}: −40% relative to basal specific [^{35}S]-GTPγS binding). This is in accordance with the results presented by Lunn et al. [33] where Sch225336 seems to be a more effective CB_2-selective inverse agonist than SR144528 (in a [^{35}S]-GTPγS assay and a forskolin-stimulated cAMP accumulation assay). However, further investigation will be necessary to confirm differences in the intrinsic activity of these two compounds.

However, Sch225336 is a potent inverse agonist which is relatively rapidly metabolized and cleared from plasma and can be useful as a short acting compound to study the inverse agonistic effect on the peripheral CB_2 receptors.

In conclusion, we have successfully synthesized [^{11}C]methoxy-Sch225336 with reasonable yields and a good radiochemical purity and specific activity. Biodistribution studies in normal mice showed only limited brain uptake limiting the potential usefulness of this tracer agent to the visualization and study of peripheral CB_2 receptor expression with PET. Further research is ongoing to validate in vivo binding of this compound to the CB_2 receptor.

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