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Characterization of [^{125}I]-SB-258585 binding to human recombinant and native 5-HT$_6$ receptors in rat, pig and human brain tissue

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1 SB-258585 (4-Iodo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzenesulphonamide) is a high affinity ligand at 5-HT$_6$ receptors. It displays over 100 fold selectivity for the 5-HT$_6$ receptor over all other 5-HT receptors tested so far. SB-258585 has been radiolabelled, to high specific activity, for its characterization as a 5-HT$_6$ receptor selective radioligand.

2 [^{125}I]-SB-258585 bound, with high affinity, to a single population of receptors in a cell line expressing human recombinant 5-HT$_6$ receptors. Kinetic and saturation binding experiments gave pK$_D$ values of 9.01 ± 0.09 and 9.09 ± 0.02, respectively.

3 In membranes derived from rat or pig striatum and human caudate putamen, [^{125}I]-SB-258585 labelled a single site with high levels (>60%) of specific binding. Saturation analysis revealed pK_D values of 8.56 ± 0.07 for rat, 8.60 ± 0.10 for pig and 8.90 ± 0.02 for human. B_{max} values for the tissues ranged from 173 ± 23 and 181 ± 25 fmol mg^{-1} protein in rat and pig striatum, respectively, to 215 ± 41 fmol mg^{-1} protein in human caudate putamen.

4 The pK_i rank order of potency for a number of compounds, determined in competition binding assays with [^{125}I]-SB-258585, at human caudate putamen membranes was: SB-271046 > SB-258585 > SB-214111 > methiothepin > clozapine > 5-Me-OT > 5-HT > Ro 04-6790 > mianserin > ritanserin = amitriptyline > 5-CT > mesulergine. Similar profiles were obtained from pig and rat striatal membranes and recombinant 5-HT$_6$ receptors; data from the latter correlated well with [^{3}H]-LSD binding.

5 Thus, [^{125}I]-SB-258585 is a high affinity, selective radioligand which can be used to label both recombinant and native 5-HT$_6$ receptors and will facilitate further characterization of this receptor subtype in animal and human tissues.

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Abbreviations: CHO, chinese hamster ovary; 5-CT, 5-carboxamidotryptamine; HEK, human embryonic kidney; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]; 5-HT, 5-hydroxytryptamine; LSD, D-lysergic acid diethylamide; 5-Me-OT, 5-methoxytryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)-tetralin; Ro 04-6790, 4-amino-N-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide; Ro 63-05636, 4-amino-N-(2,6 bis-methylamino-pyridine-4-yl)-benzene sulphonamide; SB-214111, 4-Bromo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzensulphonamide; SB-258585, 4-Iodo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzene-sulphonamide; SB-271046, 5-Chloro-3-methyl-benzo[*b*]thiophene-2-sulphonic acid (4-methoxy-3-piperazin-1-yl-phenyl)-amide

Introduction

5-Hydroxytryptamine (5-HT) exerts a wide variety of physiological and behavioural effects through actions on multiple receptor subtypes. These receptors have been classified by structural, functional and pharmacological criteria into seven distinct receptor classes (5-HT$_{1\text{-}7}$) (Hoyer *et al*., 1994; Hoyer & Martin, 1996).

The rat and human 5-HT$_6$ receptors have been cloned and characterized (Monsma *et al*., 1993; Ruat *et al*., 1993; Kohen *et al*., 1996). More recently, a splice variant of the human 5-HT$_6$ receptor has been identified and although mRNA for the truncated variant was detected in caudate and substantia nigra, *in vitro* studies have demonstrated that it is non-functional (Olsen *et al*., 1999). 5-HT$_6$ receptors couple positively to adenylyl cyclase when expressed in cell lines (Monsma *et al*., 1993; Ruat *et al*., 1993; Kohen *et al*., 1996; Boess *et al*., 1997). Functionally coupled endogenous 5-HT$_6$-like receptors have been described in mouse neuroblastoma derived cell lines (Conner & Mansour, 1990; Unsworth & Molinoff, 1994), primary cultures of mouse striatal neurons (Sebben *et al*., 1994) and in pig striatal membranes (Schoeffter & Waeber, 1994) suggesting that native 5-HT$_6$ receptors are also positively coupled to adenylyl cyclase.

In the rat brain relatively high levels of 5-HT$_6$ receptor mRNA are detected in the striatum, olfactory tubercle, nucleus accumbens, cerebral cortex and hippocampus (Monsma *et al*., 1993; Ruat *et al*., 1993; Ward *et al*., 1995). 5-HT$_6$ receptor mRNA expression is not affected by a selective lesion of serotonergic neurons (Gerard *et al*., 1996), suggesting a post-synaptic localization of the receptors. This has subsequently been demonstrated by light and electron microscopic studies using a 5-HT$_6$ receptor antiserum (Gerard *et al*., 1997), where,

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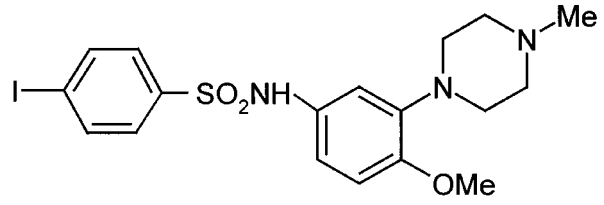
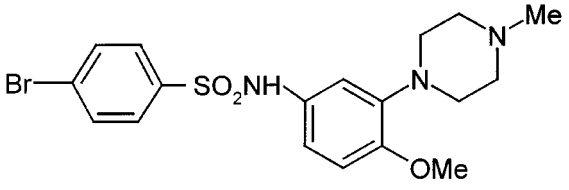
both in the striatum and hippocampus, the 5-HT$_6$ receptor-like immunoreactivity was associated with dendritic processes (Gerard *et al*., 1997). Localization of 5-HT$_6$ receptors to both basal ganglia and limbic structures suggests that this receptor subtype may participate in the serotonergic control of motor function, mood-dependent behaviour, depression and cognition. 5-HT$_6$ receptor function has been addressed in a number of recent studies using antisense oligonucleotides to reduce the number of receptors (Bourson *et al*., 1995; Yoshioka *et al*., 1998) and selective 5-HT$_6$ receptor antagonists (Sleight *et al*., 1998; Bourson *et al*., 1998). These studies suggest that the 5-HT$_6$ receptor may be involved in the modulation of cholinergic neuronal function (Bourson *et al*., 1995; 1998; Sleight *et al*., 1998; Bentley *et al*., 1999) and in increased 5-HT release induced by conditioned fear stress (Yoshioka *et al*., 1998). A study with the recently described selective 5-HT$_6$ receptor antagonist, 5-Chloro-3-methyl-benzo [*b*]thiophene-2-sulphonic acid (4-methoxy-3-piperazin-1-yl-phenyl)-amide (SB-271046) (Bromidge *et al*., 1999; Routledge *et al*., 1999) has confirmed the putative link between 5-HT$_6$ receptors and cognitive function (Rogers *et al*., 1999).

Prior to the recent introduction of [^{3}H]-4-amino-N-(2,6 bis-methylamino-pyridine-4-yl)-benzene sulphonamide ([^{3}H]-Ro 63-0563) (Boess *et al*., 1998) and [^{125}I]-SB-258585 (4-Iodo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzenesulphonamide; structure shown in Figure 1) (Hirst *et al*., 1999 and the present study), there have been no selective radioligands for the 5-HT$_6$ receptor and studies have relied on non-selective radioligands, including radiolabelled 5-HT and D-lysergic acid diethylamide (LSD). In the present study we describe the characterisation of [^{125}I]-SB-258585, a novel, selective 5-HT$_6$ receptor antagonist which binds to both recombinant and native 5-HT$_6$ receptors with high affinity and displays high levels of specific binding. A preliminary account of the data presented here has been published in abstract form (Hirst *et al*., 1999; Minton *et al*., 1999).



SB-258585



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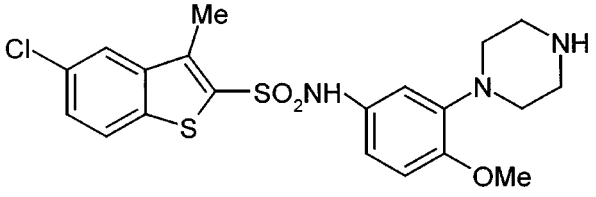


SB-271046

Figure 1 Chemical structures of SB-258585, 4-Iodo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzenesulphonamide, SB-214111, 4-Bromo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzene-sulphonamide and SB-271046, 5-Chloro-3-methyl-benzo[*b*]thiophene-2-sulphonic acid (4-methoxy-3-piperazin-1-yl-phenyl)-amide.

Methods

Preparation of membranes

Cloned receptors HeLa cells stably transfected with cDNA coding for the human 5-HT$_6$ receptor were obtained, under a licensing agreement, from Dr D. Sibley (National Institute of Health, Bethesda, MD, U.S.A.). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal bovine serum and were routinely treated with 5 mM sodium butyrate 24 h prior to harvesting. The cells were harvested in phosphate buffered saline (PBS) containing 0.1 mM EDTA and pelleted by centrifugation ($1000 \times g$), the supernatant was discarded and the pellets were stored at -80°C prior to membrane preparation. For preparation of membranes, cell pellets were homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°C), for approximately 20 s, using a Kinematic Ultra-Turrax homogenizer. The homogenates were centrifuged at $35{,}000 \times g$ for 20 min and the resulting pellet was re-homogenized and incubated at 37°C for 15 min. Following two further centrifugation steps (as above) the membranes were finally resuspended (approximately 4 mg protein ml^{-1}) and stored at -80°C until use.

Native tissue Striatal tissue from adult rats (Sprague-Dawley, 200–250 g, Charles River, U.K.), adult pigs (from a local abattoir: Dalehead Foods, Linton, U.K.) and human caudate putamen tissue (from three non-identifiable patients aged 64–76 years, whose cause of death was non-neurological, from Resource, Institute of Neurology, London, U.K. approved by a local ethics committee) were homogenized and prepared exactly as described above for the HeLa cells.

Radioligand binding

Studies using membranes of cells stably transfected with the 5-HT$_6$ receptor were carried out in a buffer containing 20 mM HEPES, 3 mM $MgCl_2$ and 2 mM ascorbate (pH 7.4). A number of experiments were carried out using the Tris based buffer, described below, with no apparent differences (data not shown). Studies using membranes derived from rat, pig or human brain tissues were carried out in a buffer containing 50 mM Tris-HCl, 10 μM pargyline, 5 mM $MgCl_2$, 5 mM ascorbate and 0.5 mM EDTA (pH 7.4).

Binding assays consisted of 50 μl of displacing compound or buffer, 400 μl of membrane suspension (corresponding to approximately 15 μg protein well^{-1} for the recombinant cells and 60 μg protein well^{-1} for the brain tissue) and 50 μl of [^{125}I]-SB-258585 (specific activity, 2000 Ci mmol^{-1}). [^{125}I]-SB-258585 was used at a concentration of 0.1 nM.

Association rates were determined by incubation of membranes with radioligand at 37°C for time periods from 0.5–120 min before termination of the experiment by filtration (described below). Dissociation rates were determined by pre-incubating membranes with [^{125}I]-SB-258585 for 45 min at 37°C, 10 μM methiothepin was then added to initiate dissociation and the experiment was terminated by filtration

after incubating for 0.5–120 min. For saturation analysis, membranes were incubated with 0.1 nM [^{125}I]-SB-258585 and unlabelled SB-258585 to give 12 final ligand concentrations ranging from approximately 0.1–25 nM, for 45 min at 37°C. In competition binding experiments, 10 concentrations of the competing ligands were tested (concentration range:0.03 nM–1 μM and 0.3 nM–10 μM) at a final [^{125}I]-SB-258585 concentration of 0.1 nM. Saturation and competition studies, using [^{3}H]-LSD as a radioligand, were also undertaken using membranes from HeLa cells expressing human recombinant 5-HT$_6$ receptors. In saturation studies eight concentrations of [^{3}H]-LSD were used (final concentrations of approximately 0.05–10 nM), whereas a single concentration of 2 nM was used in competition binding experiments.

Incubations with [^{125}I]-SB-258585 and [^{3}H]-LSD were for 45 min at 37°C (except for the kinetic studies). Non-specific binding was measured in the presence of 10 μM methiothepin. The experiments were terminated by rapid filtration through Whatman GF/B filters, pre-treated with 0.3% (v v^{-1}) polyethyleneimine (PEI), and washed with 6–9 ml of ice cold buffer. Radioactivity was determined by gamma spectrometry using a Packard Cobra II gamma counter or by liquid scintillation spectrometry using a Packard 2700 liquid scintillation counter.

To determine the relative receptor selectivity of SB-258585, binding studies were also carried out on human cloned 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_7$, dopaminergic D$_2$ and D$_3$ and adrenergic α_{1B} receptors and to native 5-HT$_4$ receptors. Brief details of the cell line, radioligands used and the compounds included to define non-specific binding and references to the methodologies are shown in Table 1.

Protein concentrations were determined using the Bradford assay method (Bio-Rad protein assay kit, Bio-Rad, York, U.K.) using bovine serum albumin as a standard.

Data analysis

Association and dissociation studies were analysed using GRAFIT (Erithacus Software Ltd., Staines, U.K.). In saturation binding studies, K_D and B_{max} values were calculated using Radlig and LIGAND (Biosoft, Cambridge, U.K.) (Munson & Rodbard, 1980; McPherson, 1997). The concentration of drug inhibiting specific radioligand binding by 50% (IC_{50}) was determined by iterative curve fitting (Bowen & Jerman, 1995). pK_i values (the negative $\log_{10}$ of the molar K_i) for receptor binding were then calculated from the IC_{50} values as described by Cheng & Prusoff (1973) using the K_D values determined in the saturation binding studies. Data are expressed as the mean ± s.e.mean of at least three separate experiments.

Materials

SB-271046 (5-Chloro-3-methyl-benzo[*b*]thiophene-2-sulphonic acid (4-methoxy-3-piperazin-1-yl-phenyl)-amide), SB-214111 (4-Bromo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzenesulphonamide), SB-258585 (4-Iodo-*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzenesulphonamide) and Ro 04-6790, 4-amino-N-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide were synthesized by SmithKline Beecham Pharmaceuticals (Harlow, U.K.); the chemical structures of SB-258585 and SB-214111 are shown in Figure 1. [^{125}I]-SB-258585 was prepared at SmithKline Beecham (Synthetic Isotope Chemistry) by reaction of the tributyltin derivative of SB-258585 with chloramine-T and sodium [^{125}I]-iodide. Methiothepin mesylate, clozapine, ritanserin, mianserin hydrochloride, 5-carboxamidotryptamine maleate (5-CT) and mesulergine hydrochloride were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). 5-hydroxytryptamine creatine sulphate (5-HT), 5-methoxytryptamine hydrochloride (5-Me-OT), amitriptyline hydrochloride and pargyline were purchased from Sigma (Poole, U.K.). Cell culture reagents were obtained from Life Technologies Ltd. (Paisley, U.K.). All other reagents were obtained from Sigma or Merck-BDH (Lutterworth, U.K.) and were of analytical grade.

Results

Receptor binding profile of SB-258585

To determine the relative receptor selectivities of SB-258585, binding studies were carried out on a number of receptors, as detailed in Table 1. SB-258585 had the highest affinity for the 5-HT$_6$ receptor (pK_i = 8.53) with greater than 100 fold selectivity over all other receptors investigated. The compound had only modest affinity for 5-HT$_{1D}$ receptor (pK_i = 6.39),

Table 1 Selectivity profile of SB-258585

Receptor	*Cell line/source*	*Radioligand*	*Concentration* (nM)	*Non-specific binding*	*References*	*SB-258585* (pKi)
5-HT$_{1A}$	HEK293	[^{3}H]-8-OH-DPAT	1	5-HT (10 μM)	5	6.19 ± 0.06
5-HT$_{1B}$	CHO	[^{3}H]-5-HT	4	5-HT (10 μM)	6,7	6.35 ± 0.02
5-HT$_{1D}$	CHO	[^{3}H]-5-HT	4	5-HT (10 μM)	6,7	6.39 ± 0.03
5-HT$_{1E}$	CHO	[^{3}H]-5-HT	4	5-HT (10 μM)	6,7	5.62 ± 0.05
5-HT$_{1F}$	CHO	[^{3}H]-5-HT	4	5-HT (10 μM)	1,6,7	6.20 ± 0.07
5-HT$_{2A}$	HEK293	[^{3}H]-ketanserin	0.5	Mianserin (10 μM)	13	5.99 ± 0.13
5-HT$_{2B}$	HEK293	[^{3}H]-5-HT	8	5-HT (10 μM)	2,8	5.53 ± 0.16
5-HT$_{2C}$	HEK293	[^{3}H]-mesulergine	0.6	Mianserin (10 μM)	13	5.94 ± 0.08
5-HT$_4$	Guinea-pig hippocampus	[^{125}I]-SB207710	0.02	SB-20-5008 (10 μM)	4	< 5
5-HT$_6$	HeLa	[^{3}H]-LSD	2	Methiothepin (1 μM)	9	8.53 ± 0.09
5-HT$_7$	HEK293	[^{3}H]-5CT	0.5	5-HT (10 μM)	12	5.47 ± 0.09
D$_2$ (long)	CHO	[^{125}H]-iodosulpride	0.1	YM-09151 (1 μM)	3,10	5.42 ± 0.10
D$_3$	CHO	[^{125}I]-iodosulpride	0.1	YM-09151 (1 μM)	1,10	6.12 ± 0.06
Adrenergic α_{1B}	CHO	[^{3}H]-prazosin	0.2	Phentolamine (10 μM)	11	5.51 ± 0.04

Data are the means ± s.e.mean of at least three experiments. Method references: 1: Adham *et al*., 1993; 2: Bonhaus *et al*., 1995; 3: Bowen *et al*., 1993; 4: Brown *et al*., 1993; 5: Gozlan *et al*., 1983; 6: Hamblin & Metcalf, 1991; 7: Heuring & Peroutka, 1987; 8: Kursar *et al*., 1992; 9: Monsma *et al*., 1993; 10: Sokoloff *et al*., 1992; 11: Testa *et al*., 1993; 12: To *et al*., 1995; 13: Wood *et al*., 1995.

lower affinities were seen with the 5-HT$_{1B}$ (pK_i = 6.35), 5-ht$_{1F}$ (pK_i = 6.20), 5-HT$_{1A}$ (pK_i = 6.19) and the dopamine D$_3$ (pK_i = 6.12) receptors. SB-258585 had pK_i values less than 6 at all other receptors examined (Table 1).

[^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors

Specific [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors reached equilibrium within 45 min (Figure 2). Kinetic analysis of the binding revealed a monophasic apparent association rate k_{obs} of 0.07 ± 0.014 min^{-1}. This binding was reversible and dissociation rates were determined in separate experiments by the addition of 10 μM methiothepin, giving a monophasic dissociation curve with a rate constant (k_{-1}) of 0.065 ± 0.004 min^{-1} (Figure 2). The calculated association rate (k_1) was 0.066 ± 0.01 min^{-1} nM^{-1} which gave a derived dissociation constant ($K_D = k_{-1}/k_1$) of 0.98 nM. Saturation analysis of [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors in HeLa cell membranes revealed a single binding site (Figure 3) with a K_D of 0.80 ± 0.05 nM and a B$_{max}$ of 6.1 ± 0.95 pmol mg^{-1} protein. At a radioligand concentration of 0.1 nM, the concentration used in both kinetic and competition binding experiments, specific binding represented 97 ± 0.2% of total binding. Saturation analysis of [^{3}H]-LSD binding to the same membranes gave a K_D of 1.5 ± 0.1 nM and a B$_{max}$ of 3.9 ± 0.8 pmol mg^{-1} protein (data not shown, $n = 4$).

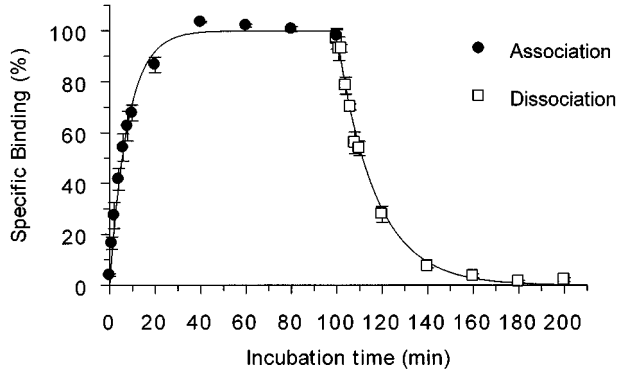


Figure 2 Association and dissociation kinetics of 0.1 nM [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors at 37°C. The apparent association rate (k_{obs}) was 0.072 ± 0.014 min^{-1}. Dissociation rate was determined in separate experiments by the addition of 10 μM methiothepin, giving a monophasic dissociation curve with a rate constant (k_{-1}) of 0.065 ± 0.004 min^{-1}. The calculated association rate (k_1) was 0.066 ± 0.01 min^{-1} nM^{-1} and the derived K_D was 0.98 nM. Data points represent the means ± s.e.mean of 3–5 independent experiments, each performed in triplicate.

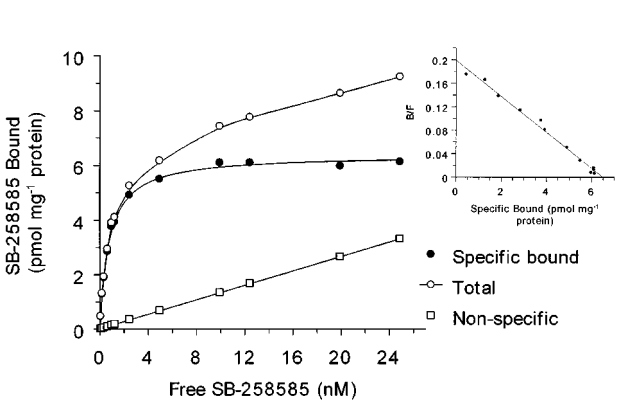
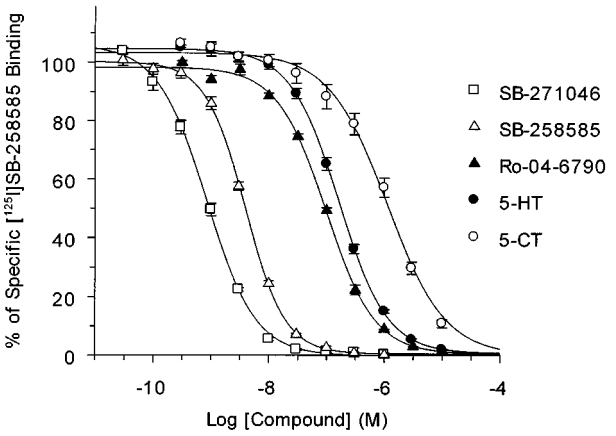


Figure 3 Saturation and Scatchard analysis of specific [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors. Membranes were incubated with 0.1 nM [^{125}I]-SB-258585 and unlabelled SB-258585 to give final ligand concentrations of approximately 0.1–25 nM for 45 min at 37°C. Non-specific binding was determined in the presence of 10 μM methiothepin. The data shown are from one of five similar experiments, each performed in triplicate. Average K_D, B$_{max}$ and standard error values are given in Table 3.

Table 2 Pharmacological profile of [^{125}I]-SB-258585 and [^{3}H]-LSD binding to human recombinant 5-HT$_6$ receptors

Displacing ligands	*[^{125}I]-SB-258585* (pK_i)	*[^{3}H]-LSD* (pK_i)
SB-271046	9.09 ± 0.07	8.92 ± 0.04
SB-258585	8.60 ± 0.08	8.53 ± 0.09
Methiothepin	8.55 ± 0.07	8.49 ± 0.21
SB-214111	8.23 ± 0.06	7.97 ± 0.11
Clozapine	7.79 ± 0.09	7.87 ± 0.18
5-Me-OT	7.10 ± 0.03	7.25 ± 0.11
Ro 04-6790	7.04 ± 0.02	6.98 ± 0.07
Mianserin	6.87 ± 0.05	7.10 ± 0.15
Ritanserin	6.89 ± 0.05	6.86 ± 0.22
Amitryptyline	6.85 ± 0.01	6.93 ± 0.15
5-HT	6.79 ± 0.02	6.97 ± 0.12
5-CT	5.91 ± 0.03	5.91 ± 0.13
Mesulergine	5.90 ± 0.03	6.11 ± 0.12

Data are means ± s.e.mean from 4–12 experiments.



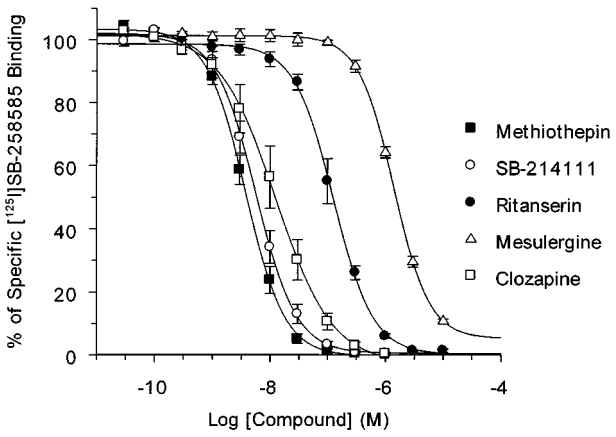


Figure 4 Pharmacological profile of [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors. Competition experiments with SB-271046, SB-258585, Ro 04-6790, 5-HT, 5-CT, methiothepin, clozapine, SB-214111, ritanserin and mesulergine were performed as described in the Methods section. Data points represent the means ± s.e.mean of 4–12 independent experiments. Average pK_i and standard error values are given in Table 2.

Pharmacological profile of [125I]-SB-258585 and [3H]-LSD binding to human recombinant 5-HT6 receptors

A comparison of the affinity of previously characterized serotonergic agonists and antagonists and novel 5-HT$_6$ receptor selective antagonists to compete with [^{125}I]-SB-258585 and [^{3}H]-LSD binding to human recombinant 5-HT$_6$ receptors is shown in Table 2 and Figure 4. The rank order of affinity to inhibit [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors, SB-271046 > SB-258585 > methiothepin > SB-214111 > clozapine > 5-Me-OT > Ro-04-6790 > mianserin > ritanserin > amitriptyline > 5-HT > 5-CT > mesulergine, was similar to that determined with [^{3}H]-LSD in the same cells (Table 2). This is shown in Figure 5 where a plot of the pK_i values determined in the competition binding experiments with both radioligands gave a correlation coefficient, r, of 0.99.

[125I]-SB-258585 binding to rat and pig striatal membranes and to human caudate putamen membranes

Specific [^{125}I]-SB-258585 binding to human caudate putamen membranes reached equilibrium within 45 min (Figure 6).

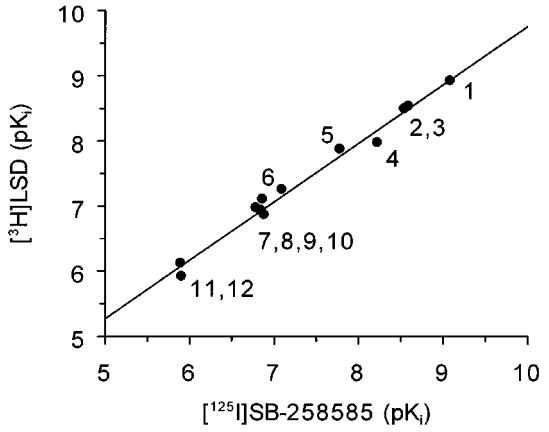


Figure 5 Correlation plot of pK_i values for 12 compounds inhibiting [^{125}I]-SB-258585 or [^{3}H]-LSD binding to human recombinant 5-HT$_6$ receptors. 1: SB-271046, 2: SB-258585, 3: methiothepin, 4: SB-214111, 5: clozapine, 6 : 5-Me-OT, 7: ritanserin, 8: mianserin, 9: amitriptyline, 10 : 5-HT, 11 : 5-CT, 12: mesulergine. Correlation coefficient, r, = 0.99 and slope = 0.90.

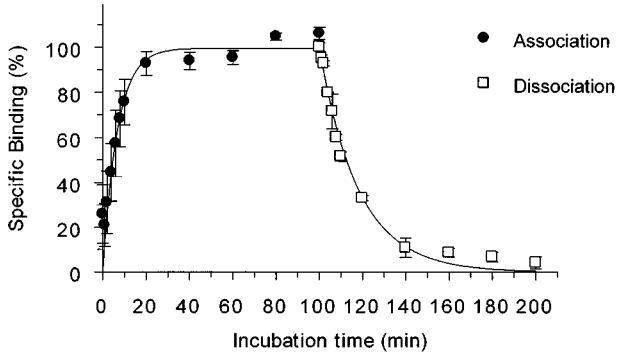


Figure 6 Association and dissociation kinetics of 0.1 nM [^{125}I]-SB-258585 binding to human caudate putamen membranes at 37°C. The apparent association rate constant (k_{obs}) was 0.064 ± 0.002 min^{-1}. Dissociation rate was determined in separate experiments by the addition of 10 μM methiothepin, giving the monophasic dissociation rate constant (k_{-1}) of 0.057 ± 0.006 min^{-1}. The calculated association rate (k_1) was 0.07 ± 0.04 min^{-1} nM^{-1} and the derived K_D was 0.80 nM. Data points represent the means ± s.e.mean of three independent experiments, each performed in triplicate.

Kinetic analysis of the binding revealed a monophasic apparent association rate k_{obs} of 0.064 ± 0.002 min^{-1}. This binding was reversible and dissociation rates, determined as described above, gave a monophasic dissociation curve with a rate constant (k_{-1}) of 0.057 ± 0.006 min^{-1} (Figure 6). The calculated association rate (k_1) was 0.07 ± 0.04 min^{-1} nM^{-1} and the derived dissociation constant ($K_D = k_{-1}/k_1$) was 0.80 nM. Figure 7 shows representative saturation curves and Scatchard analyses of specific [^{125}I]-SB-258585 binding to rat and pig striatal membranes and human caudate putamen membranes. 0.1 nM [^{125}I]-SB-258585 labelled a single binding

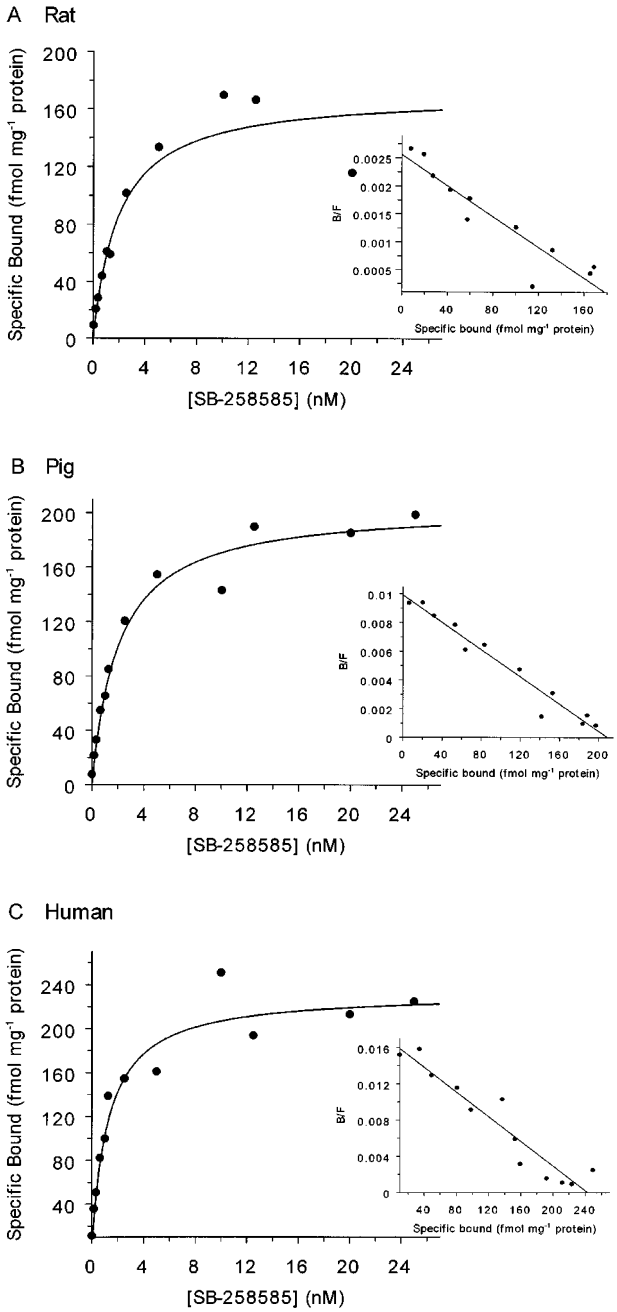


Figure 7 Saturation and Scatchard analysis of specific [^{125}I]-SB-258585 binding to rat striatal membranes (A), pig striatal membranes (B) and human caudate putamen membranes (C). Membranes were incubated with 0.1 nM [^{125}I]-SB-258585 and unlabelled SB-258585 to give final ligand concentrations of approximately 0.1 – 25 nM for 45 min at 37°C. Non-specific binding was determined in the presence of 10 μM methiothepin. The data shown are from one of 3 – 4 similar experiments, each performed in triplicate. Average K_D, B_{max} and standard error values are given in Table 3.

site, with high levels of specific binding: 59.7±0.9%, 65.5±0.7% and 67.5±1.8%, in rat, pig and human membranes, respectively. However, at higher concentrations of radioligand the specific signal decreased, for example, in the human caudate putamen membranes the specific binding was 39% at 0.5 nM and 25% at 1 nM. The binding affinities (K_D) and capacities (B_{max}) for each of the tissues, together with data from the recombinant cell line expressing human 5-HT$_6$ receptors are given in Table 3. All three tissues displayed similar 5-HT$_6$ receptor affinities for [^{125}I]-SB-258585 and similar receptor densities.

Table 3 [^{125}I]-SB-258585 binding to striatal membranes and human recombinant 5-HT$_6$ receptors

	K_D (nM)	B_{max} (fmol mg^{-1} protein)
Rat striatum	2.8±0.4	173±22
Pig striatum	2.8±0.7	181±25
Human caudate	1.3±0.04	215±41
HeLa cells	0.8±0.05	6100±950

Data are means±s.e.mean from 3–5 independent experiments each performed in triplicate.

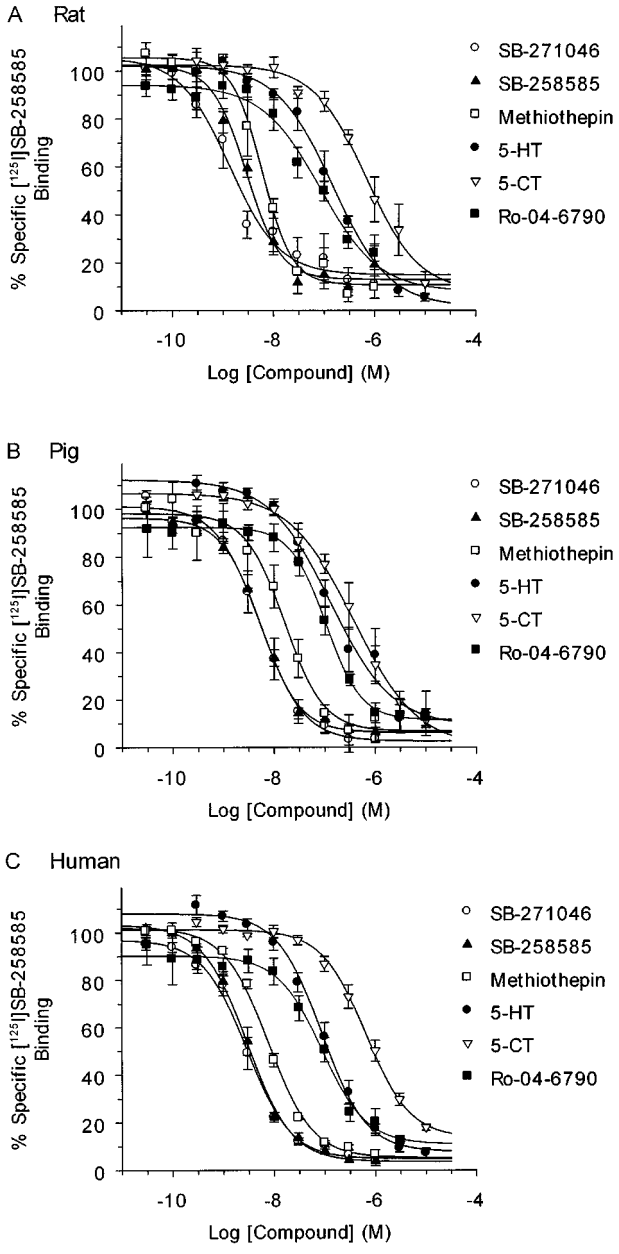


Figure 8 Pharmacological profile of [^{125}I]-SB-258585 binding to rat striatal membranes (A), pig striatal membranes (B) and human caudate putamen membranes (C). Competition experiments with SB-271046, SB-258585, methiothepin, 5-HT, 5-CT and Ro 04–6790 were performed as described in the Methods section. Data points represent the means±s.e.mean of 4–12 independent experiments. Average pK_i and standard error values are given in Table 4.

Pharmacological profile of [^{125}I]-SB-258585 binding to rat and pig striatal membranes and to human caudate putamen membranes

Competition binding analysis was used to characterize the [^{125}I]-SB-258585 binding sites in rat and pig striatal membranes and to human caudate putamen membranes (Figure 8 and Table 4). The same 12 compounds, used to profile the radioligand binding to recombinant receptors, were used in the experiments with native tissues. The pK_i rank order of potency at human caudate putamen membranes was SB-271046 > SB-258585 > SB-214111 > methiothepin > clozapine > 5-Me-OT > 5-HT > Ro-04-6790 > mianserin > ritanserin = amitriptyline > 5-CT > mesulergine. Similar values were obtained from rat and pig striatal membranes (Table 4). A comparison of the pK_i values determined using human recombinant 5-HT$_6$ receptors and human caudate putamen membranes revealed a statistically significant correlation, $r=0.98$, (Figure 9), indicating that the native tissue [^{125}I]-SB-258585 binding site had a pharmacological profile consistent with that of the 5-HT$_6$ receptor.

Discussion

This study has shown that [^{125}I]-SB-258585 is a high affinity, reversible radioligand at 5-HT$_6$ receptors. SB-258585 is a potent antagonist at 5-HT$_6$ receptors (Bromidge *et al*., 1999) and has greater than 100 fold selectivity for 5-HT$_6$ receptors over 10 other 5-HT receptors investigated. The structurally related compound, SB-271046, was shown to be more than 200 fold selective for the human 5-HT$_6$ receptor as compared to 55 other receptors, enzymes and ion channels (Routledge *et al*., 1999) and SB-271046 fully displaced specific [^{125}I]-SB-258585

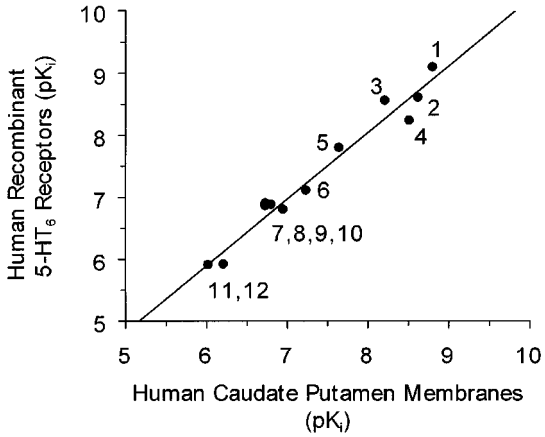


Figure 9 Correlation plot of pK_i values for 12 compounds inhibiting [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors and human caudate putamen membranes. 1: SB-271046, 2: SB-258585, 3: methiothepin, 4: SB-214111, 5: clozapine, 6: 5-Me-OT, 7: ritanserin, 8: mianserin, 9: amitriptyline, 10: 5-HT, 11: 5-CT, 12: mesulergine. Correlation coefficient, r, = 0.98 and slope = 1.08.

Table 4 Pharmacological profile of [^{125}I]-SB-258585 binding to rat striatal, pig striatal and human caudate putamen membranes

Displacing ligands	*Rat striatal membranes*	*Pig striatal membranes*	*Human caudate membranes*	*Human recombinant 5-HT$_6$ receptors*
SB-271046	9.02±0.14	8.55±0.10	8.81±0.10	9.09±0.07
SB-258585	8.59±0.09	8.41±0.05	8.63±0.07	8.60±0.08
Methiothepin	8.28±0.11	7.94±0.09	8.22±0.10	8.55±0.07
SB-214111	8.33±0.23	8.21±0.08	8.52±0.06	8.23±0.06
Clozapine	7.84±0.07	7.51±0.10	7.65±0.04	7.79±0.09
5-Me-OT	7.20±0.19	7.33±0.08	7.24±0.09	7.10±0.03
Ro 04-6790	7.31±0.08	7.00±0.09	7.03±0.07	7.04±0.02
Ritanserin	6.98±0.06	6.67±0.05	6.74±0.09	6.89±0.05
Mianserin	6.93±0.06	7.22±0.05	6.81±0.06	6.87±0.05
Amitriptyline	7.01±0.06	6.54±0.08	6.74±0.07	6.85±0.01
5-HT	6.96±0.07	7.07±0.06	6.96±0.09	6.79±0.02
5-CT	6.24±0.12	6.34±0.15	6.22±0.09	5.91±0.03
Mesulergine	5.95±0.05	6.39±0.03	6.03±0.07	5.90±0.03

Data are means±s.e.mean from 6–12 experiments.

binding in the striatal membranes. Taken together these data suggest that [^{125}I]-SB-258585 is a highly selective radioligand.

Kinetic analyses, demonstrating rapid rates of association and dissociation, and saturation binding studies show that [^{125}I]-SB-258585 binds with sub-nanomolar affinity (K_D=0.80–0.98 nM) to a single binding site in membranes prepared from HeLa cells recombinantly expressing human 5-HT$_6$ receptors. The number of binding sites labelled with [^{125}I]-SB-258585 in this cell line (6.1 pmol mg^{-1} protein) is slightly higher than that determined with a non-selective agonist radioligand, [^{3}H]-LSD (3.9 pmol mg^{-1} protein), which could be explained by an antagonist radioligand labelling all the receptors compared to the agonist radioligand which selectively binds to receptors in their high affinity state. However, there is no suggestion of multiple affinity states from either [^{3}H]-LSD saturation studies (data not shown) nor from agonist displacement curves in competition binding experiments using [^{125}I]-SB-258585 where the Hill coefficient for 5-HT, 5-CT and 5-Me-OT were 1.04±0.01, 1.05±0.07 and 0.97±0.02, respectively. Hence the minor discrepancy in the number of binding sites detected by the two different radioligands may be due to experimental variation as a result of the different approaches used in generating the saturation isotherms; increasing concentrations of [^{3}H]-LSD, from 0.05–10 nM, and a single concentration of [^{125}I]-SB-258585 (0.1 nM) and unlabelled SB-258585 to give 12 final ligand concentrations ranging from approximately 0.1–25 nM.

The pharmacological profile of [^{125}I]-SB-258585, determined in competition binding studies, at human 5-HT$_6$ receptors stably expressed in HeLa cells correlated closely to the profile determined with [^{3}H]-LSD (r=0.99). This profile was also consistent with previous studies on cloned rat (Monsma *et al*., 1993; Boess *et al*., 1997) and human (Kohen *et al*., 1996) 5-HT$_6$ receptors using [^{125}I]-LSD, [^{3}H]-LSD or [^{3}H]-5-HT and also with a more recent study which reported on the characterization of a selective 5-HT$_6$ receptor radioligand, [^{3}H]-Ro 63-0563 (Boess *et al*., 1998).

In the majority of studies to date, non-selective radioligands, including [^{3}H]-5-HT, [^{3}H]-LSD, [^{125}I]-LSD and [^{3}H]-clozapine, have been used to label 5-HT$_6$ receptors. In simple, recombinant systems these ligands are adequate (Monsma *et al*., 1993; Kohen *et al*., 1996; Boess *et al*., 1997), however their lack of selectivity has precluded definitive studies on 5-HT$_6$ receptors in native tissues. For example, studies investigating the effects of antisense oligonucleotides on 5-HT$_6$ receptor expression (Bourson *et al*., 1995; Yoshioka *et al*., 1998) have used [^{3}H]-LSD binding. Addition of compounds to prevent binding of [^{3}H]-LSD to other receptors may have resulted in an under or over estimation in the number of 5-HT$_6$ receptors. [^{3}H]-clozapine has also been used to label 5-HT$_6$ receptors in rat brain (Glatt *et al*., 1995). However under the conditions employed this radioligand was shown to label at least two populations of receptors, one of which was probably a muscarinic receptor (Glatt *et al*., 1995). [^{3}H]-Ro 63-0563 selectively binds to 5-HT$_6$ receptors in recombinant cell lines (Boess *et al*., 1998). However, in binding assays, using [^{3}H]-Ro 63-0563, with rat and pig striatal membranes there are relatively high levels of non-specific binding (70–90%) (Boess *et al*., 1998), which could make detailed investigations of 5-HT$_6$ receptor localization and pharmacology difficult, particularly in human brain tissues, where post-mortem delays and protein degradation could reduce this small specific binding signal further.

High levels of 5-HT$_6$ mRNA have been demonstrated in rat striatum by Northern blot analysis (Monsma *et al*., 1993; Ruat *et al*., 1993), *in situ* hybridization (Ruat *et al*., 1993; Ward *et al*., 1995) and by reverse transcriptase-polymerase chain reaction (RT–PCR) (Gerard *et al*., 1996). 5-HT$_6$ receptor-like immunoreactivity has also been shown, in addition to other regions, in the rat striatum (Gerard *et al*., 1997) and 5-HT$_6$ receptor binding sites have been identified in rat and pig striatal membranes (Boess *et al*., 1998). Based on these previous studies, we used striatal membranes to investigate and characterize 5-HT$_6$ receptor binding sites in native brain tissue. The amount of specific binding observed in rat and pig striatal membranes and in human caudate putamen membranes, at a radiolabel concentration of 0.1 nM, was 59.7±0.9%, 65.5±0.7% and 67.5±1.8%, respectively. These values are considerably higher than those reported with [^{3}H]-Ro 63-0563, which were 10–20% and 20–30% for rat and pig tissue, respectively (Boess *et al*., 1998). The higher specific binding with [^{125}I]-SB-258585 allowed a detailed characterization of radioligand binding in the rat and pig striatal membranes and in human caudate putamen membranes. Kinetic studies on specific [^{125}I]-SB-258585 binding to human caudate putamen membranes gave results which were comparable to those obtained for the human 5-HT$_6$ receptors expressed in a HeLa cell line. In the human caudate putamen membranes the binding was high affinity, saturable and reversible and the K_D value determined from kinetic studies was 0.80 nM. In equilibrium binding studies, [^{125}I]-SB-258585 labelled a single binding site in the native tissues with a K_D of 1.3 nM for human caudate putamen membranes and 2.8 nM for rat and pig striatal membranes, which correlated well with the affinity estimate from the kinetic studies. The number of binding sites labelled with [^{125}I]-SB-258585 in the native tissues

was in the region of 170–215 fmol mg^{-1} protein, depending on the species (Table 3), a value similar to that previously determined with [^{3}H]-Ro 63-0563 in pig striatal membranes (Boess *et al*., 1998).

The pharmacological profile, determined in the present study with both previously characterized serotonergic agonists and antagonists and novel 5-HT$_6$ receptor selective antagonists, did not show any notable differences between rat, pig or human membranes (Table 4), suggesting that the pharmacology of the 5-HT$_6$ receptor is conserved between species. The profile was also similar to that obtained for the human 5-HT$_6$ receptor stably expressed in a cell line (Figure 9). Collectively these data confirm that [^{125}I]-SB-258585 is selectively labelling 5-HT$_6$ receptors in native tissues. These results provide the first data describing the pharmacology of the 5-HT$_6$ receptor in membranes prepared from human caudate putamen.

Several studies have described a clear association of 5-HT$_6$ receptors (Ruat *et al*., 1993; Monsma *et al*., 1993; Ward *et al*., 1995; Kohen *et al*., 1996; Gerard *et al*., 1997) with both the basal ganglia and the limbic system, suggesting potential roles for this receptor in control of motor function, mood dependent behaviour, depression and cognition. Furthermore, the high affinity of classical and atypical antipsychotics (Monsma *et al*., 1993; Roth *et al*., 1994; Kohen *et al*., 1996), together with the localization of the receptor in cortical and limbic areas suggests that 5-HT$_6$ receptors may play a role in the pathophysiology of schizophrenia. Targeting 5-HT$_6$ receptors may offer the potential of antipsychotic activity, without the propensity for extrapyramidal side effects associated with dopamine D$_2$ receptor blockade. [^{125}I]-SB-258585 will be a useful tool in studying 5-HT$_6$ receptor density and distribution in animal models of disease and in human brains from patients suffering from schizophrenia and Alzheimer's disease. Preliminary studies have demonstrated that [^{125}I]-SB-258585 is also a suitable ligand for autoradiographic labelling of 5-HT$_6$ receptors in brain sections (Roberts *et al*., 1999).

In conclusion, we have described the characterization of a radioligand, [^{125}I]-SB-258585, which selectively binds to recombinant and native 5-HT$_6$ receptors with high affinity. A comparison of the pharmacological profile of [^{125}I]-SB-258585 binding to human recombinant 5-HT$_6$ receptors and native tissues demonstrates the similarity in 5-HT$_6$ receptor pharmacology between species. This is the first radioligand binding study to characterize and quantify 5-HT$_6$ receptors in human brain tissue.

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