British Journal of Pharmacology (2000) 130, 1606–1612

www.nature.com/bjp

Characterization of SB-271046: A potent, selective and orally active 5-HT_6 receptor antagonist

*,[1]Carol Routledge, [2]Steven M. Bromidge, [2]Stephen F. Moss, [1]Gary W. Price, [1]Warren Hirst, [1]Helen Newman, [1]Graham Riley, [1]Tracey Gager, [1]Tania Stean, [1]Neil Upton, [3]Stephen E. Clarke, [1]Anthony M. Brown & [1]Derek N. Middlemiss

[1]Department of Neuroscience Research, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW; [2]Department of Discovery Chemistry, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW and [3]Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW

1 SB-271046, potently displaced [^{3}H]-LSD and [^{125}I]-SB-258585 from human 5-HT_6 receptors recombinantly expressed in HeLa cells *in vitro* (pK_i 8.92 and 9.09 respectively). SB-271046 also displaced [^{125}I]-SB-258585 from human caudate putamen and rat and pig striatum membranes (pK_i 8.81, 9.02 and 8.55 respectively).

2 SB-271046 was over 200 fold selective for the 5-HT_6 receptor vs 55 other receptors, binding sites and ion channels.

3 In functional studies on human 5-HT_6 receptors SB-271046 competitively antagonized 5-HT-induced stimulation of adenylyl cyclase activity with a pA_2 of 8.71.

4 SB-271046 produced an increase in seizure threshold over a wide-dose range in the rat maximal electroshock seizure threshold (MEST) test, with a minimum effective dose of $\leqslant$0.1 mg kg^{-1} p.o. and maximum effect at 4 h post-dose. The level of anticonvulsant activity achieved correlated well with the blood concentrations of SB-271046 (EC_{50} of 0.16 μM) and brain concentrations of 0.01–0.04 μM at C_{max}.

5 These data, together with the observed anticonvulsant activity of other selective 5-HT_6 receptor antagonists, SB-258510 (10 mg kg^{-1}, 2–6 h pre-test) and Ro 04-6790 (1–30 mg kg^{-1}, 1 h pre-test), in the rat MEST test, suggest that the anticonvulsant properties of SB-271046 are likely to be mediated by 5-HT_6 receptors.

6 Overall, these studies demonstrate that SB-271046 is a potent and selective 5-HT_6 receptor antagonist and is orally active in the rat MEST test. SB-271046 represents a valuable tool for evaluating the *in vivo* central function of 5-HT_6 receptors.

British Journal of Pharmacology (2000) **130,** 1606–1612

Keywords: Human 5-HT_6 receptor; SB-271046; [^{3}H]-LSD; SB-258585; adenylyl cyclase; rat maximal electroshock seizure threshold (MEST) test; pharmacokinetics

Abbreviations: 5-HT, 5-hydroxytryptamine creatinine sulphate; LSD, D-lysergic acid diethylamide; MEST, maximal electroshock seizure threshold; Ro 04-6790, 4-amino-*N*-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide; SB-258510, *N*-[4-Methoxy-3-(4-methyl-1-piperazinyl)-phenyl]-5-chloro-3-methylbenzo-thiophene-2-yl sulphonamide monohydrochloride; 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 monohydrochloride

Introduction

The rat 5-HT_6 receptor was first identified in 1993 by Ruat *et al*. (1993) and Monsma *et al*. (1993) and more recently the human 5-HT_6 receptor gene was cloned and characterized (Kohen *et al*., 1996). Since then, a number of publications have suggested a role for 5-HT_6 receptor antagonists in the treatment of schizophrenia, depression and cognitive impairment. A role for this receptor in the treatment of schizophrenia and depression is supported by its distribution in the brain and the high affinity of therapeutic atypical antipsychotics, particularly clozapine, and antidepressants for the 5-HT_6 receptor (Monsma *et al*., 1993, Ruat *et al*., 1993; Roth *et al*., 1994). Other evidence pointing towards a role for this receptor in anxiety/stress/depression came from a study using antisense oligonucleotides directed at 5-HT_6 receptor mRNA. Central administration of 5-HT_6 specific antisense oligonucleotides abolished the conditioned fear stress-induced increase in 5-HT release (Yoshioka *et al*., 1998). In addition, blockade of endogenous corticosterone synthesis modulates 5-HT_6 receptor mRNA in specific areas of the rat hippocampus (Yau *et al*., 1997). However, data from the majority of *in vivo* studies point to a role for the 5-HT_6 receptor in the modulation of cholinergic neuronal activity and, perhaps as a consequence of this, cognitive function. Thus, central administration of 5-HT_6 specific antisense oligonucleotides induced a behavioural syndrome which was dose-dependently blocked by the muscarinic receptor antagonist atropine (Bourson *et al*., 1995), suggesting that an increase in central cholinergic neuronal activity may be a consequence of 5-HT_6 receptor blockade. These data were supported by a study using the selective 5-HT_6 receptor antagonist Ro 04-6790 (Sleight *et al*., 1998), where Ro 04-6790 elicited some of the components of the above behavioural syndrome (Sleight *et al*., 1998; Bentley

*Author for correspondence;
E-mail: Carol_Routledge-1@sbphrd.com

et al., 1999). Ro 04-6790 has also been shown to block scopolamine-induced rotations in 6-OH-DA-lesioned rats, an effect suggested to support the modulation of the cholinergic system by 5-HT$_6$ receptor antagonists (Bourson *et al*., 1998). In addition, central administration of the same antisense oligonucleotide was shown to increase cognitive performance in the Morris water maze (Bentley *et al*., 1997). Despite the first identification of 5-HT$_6$ receptors in 1993, there are relatively few studies demonstrating the *in vivo* function of this receptor, which is probably due to the lack of suitable pharmacological tools. The development of the selective 5-HT$_6$ receptor antagonist, Ro 04-6790, was a significant improvement over antisense treatment, however, whilst this compound was highly selective for the 5-HT$_6$ receptor, it had moderate affinity (pK_i 7.4) and $<1\%$ CNS penetration (Sleight *et al*., 1998).

With the aim of further elucidating the *in vivo* function of the 5-HT$_6$ receptor, we have identified a potent, selective and orally active 5-HT$_6$ receptor antagonist, SB-271046 (Bromidge *et al*., 1999), and report here on its *in vitro* and *in vivo* characterization. Recent studies using this compound provide initial supportive evidence for the role of the 5-HT$_6$ receptor in cognitive enhancement (Rogers *et al*., 1999). A preliminary account of the data presented here has been published in abstract form (Routledge *et al*., 1999; Stean *et al*., 1999).

Methods

Radioligand binding studies

Cell line The human 5-HT$_6$ serotonin receptor stably expressed in HeLa cells was obtained from Dr David Sibley of the Molecular Neuropharmacology Section, Experimental Therapeutics Branch, Public Health Service, National Institutes of Health, Bethesda, MA, 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.

Radioligand binding Radioligand binding was carried out as described (Hirst *et al*., 2000). In brief, radioligand binding was performed on membranes from HeLa cells stably transfected with the human 5-HT$_6$ receptor (see above) and 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 tissues (from non-identifiable patients aged 64–76 years, whose cause of death was non-neurological, from Resource, Institute of Neurology, London, U.K.). Membranes were incubated with 1 nM [^{125}I]-SB-258585 (Hirst *et al*., 2000) or 2 nM [^{3}H]-LSD for 45 min at 37°C. Non-specific binding was defined by the inclusion of 10 μM methiothepin and the assay was terminated by rapid filtration through Whatman GF/B filters.

For receptor selectivity studies on other 5-HT receptors, details of the radioligands used and assay conditions are given in Hirst *et al*. (2000). SB-271046 was also tested in a further 55 binding assays by CEREP (Le Bois l'Eveque, 86600 Celle L'Evescault, France) (CEREP Task Order 882035).

Adenylyl cyclase measurements

Membrane preparation Cell pellets were stored at −80°C prior to membrane preparation. The cell pellet was thawed at room temperature, and divided into eight volumes, each of which was homogenized in 30 mls ice-cold HEPES buffer (20 mM HEPES, 10 mM $MgSO_4$, pH 7.4) by polytron homogenization. Homogenates were then centrifuged at 18,000 r.p.m. ($35{,}000 \times g$) for 10 min. The supernatant was discarded and the pellet resuspended in 30 mls of HEPES buffer. Homogenates were then incubated at 37°C for 20 min to allow the breakdown of endogenous 5-HT. The suspension was washed a further two times by centrifugation before resuspension at a cell density of 1×10^8 cell ml^{-1}, and storage at −80°C.

Method

Adenylyl cyclase activity in HeLa cells transfected with the human 5-HT$_6$ receptor (as described above) was determined by measuring the conversion of [α-^{33}P]-ATP to [^{33}P]-cAMP. [α-^{33}P]-cAMP production is small ($<1\%$) as a proportion of the [α-^{33}P]-ATP added and so must be efficiently separated from unconverted [α-^{33}P]-ATP by Dowex-Alumina chromatography using a dual column separation method adapted from Salomon (1979). The assay mixture consisted of 50 mM Tris HCl buffer containing: $MgCl_2$ (5 mM), GTP (50 μM), ATP (100 μM), phosphocreatine (20 mM), creatine phosphokinase (40 units ml^{-1}), myokinase (50 units ml^{-1}), and 1-methyl-3-isobutylxanthine (IBMX) (0.5 μM), in a total assay volume of 50 μl. Incubations (37°C, 10 min) were started by addition of 20 ml of membrane suspension (20–25 mg protein) to tubes containing incubation buffer, [α-^{33}P]-ATP (1–1.5 μCi tube^{-1} sp. act. 2000 Ci mmol^{-1}) and test drugs as appropriate. The incubation was stopped by addition of 100 μl of 0.5 M HCl containing ATP (40 mM), cyclic AMP (10 mM) and [^{3}H]-cAMP ($\sim$10,000 d.p.m. specific activity 27 Ci mmol^{-1}) for calculation of column recovery. Samples were counted in Ultima-XR scintillant using a dual label protocol and the tritium signal was used to correct for per cent column recovery.

Maximal electroshock seizure threshold (MEST) test

All experimental work was conducted in compliance with the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986, and was reviewed and approved by the SmithKline Beecham Procedures Review Panel.

Male Sprague Dawley rats (100–150 *g*), supplied by Charles River, U.K. were housed in groups of 10 at a room temperature of 20–22°C. Animals were maintained on a 12 h light/dark cycle with lights on between 0600 and 1800 h Food (Combined Rat and Mouse Diet, Special Diet Services, Witham, U.K.) and water were available *ad libitum*. Drug treatments were evaluated between 1400 and 1800 h alongside time-matched vehicle-treated controls.

The threshold current for electroshock-induced tonic hindlimb extensor seizure was determined using a Hugo Sachs Electronik stimulator (Germany), which delivered an adjustable constant current (1–300 mA) of 0.3 s duration, 50 Hz, sinewave form, *via* corneal electrodes. The stimulus intensity was varied, from a typical baseline of 25 mA, by an 'up and down' method of shock titration (see Upton *et al*., 1997, for details). Data generated from treatment groups of $n = 11–14$ were used to calculate the seizure threshold (current producing tonic hindlimb extensor seizure in 50% of animals) $\pm$ s.e. values according to the method of Kimball *et al*. (1957). Elevation of seizure threshold is indicative of an anticonvulsant effect whereas a reduction in seizure threshold is indicative of proconvulsant activity. The effects of the selective 5-HT$_6$ receptor antagonists, SB-271046 (0.1–30 mg kg^{-1} p.o., 4 h pre-test), SB-258510 (10 mg kg^{-1} p.o., 2–6 h pre-test) (Bromidge *et al*., 1999) and Ro 04-6790 (0.3–30 mg kg^{-1} i.p.,

1 h pre-test) (Sleight *et al*., 1998), on seizure threshold were determined. The doses of Ro 04-6790 selected for this study cover the range previously reported to evoke a number of behavioural effects in rats (Sleight *et al*., 1998; Bentley *et al*., 1999). SB-271046 and SB-258510 were suspended in 1% methyl cellulose in water and Ro 04-6790 was dispersed in saline. A 1 ml kg^{-1} dose volume was used for all treatments and doses are expressed as free base.

In order to evaluate the relationship between the level of anticonvulsant activity achieved and blood concentration, the duration of action of a high submaximal dose (10 mg kg^{-1} p.o.) of SB-271046 in the rat MEST test was evaluated in detail over a 24 h period. Following the conclusion of this study, whole brain and blood samples were taken from randomly selected animals ($n=5$) at 13 different timepoints. Samples were assayed for SB-271046 using a method based on protein precipitation with acetonitrile, followed by LC/MS/MS analysis employing positive-ion electrospray ionization, with a lowest limit of quantification (LLQ) of 0.01 μM.

Materials

SB-271046 (5-Chloro-3-methyl-benzo[*b*]thiophene-2-sulphonic acid (4-methoxy-3-piperazin-1-yl-phenyl)-amide monohydrochloride), SB-258510 (*N*-[4-Methoxy-3-(4-methyl-1-piperazinyl)-phenyl]-5-chloro-3-methylbenzo-thiophene-2-yl sulphonamide monohydrochloride) 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 structure of SB-271046 is 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. [^{3}H]-LSD, [α-^{33}P]-ATP and [^{33}P]-cAMP were obtained from NEN Du Pont. 5-hydroxytryptamine creatine sulphate (5-HT), amitriptyline hydrochloride and pargyline were purchased from Sigma (Poole, U.K.). Mesulergine hydrochloride were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). Chromatography columns and Dowex resins were obtained from Bio-rad. 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.

Data analysis

The concentration of drug inhibiting specific radioligand by 50% (IC_{50}) was determined by iterative curve fitting (Bowen & Jerman, 1995) and pK_i values (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 K_D values determined in the stauration binding studies. Data are expressed as the mean$\pm$s.e.mean of at least three separate experiments.

Drug concentration-response curves from adenylyl cyclase assays were fitted to a 4-parameter logistic equation (GRAFIT, Erithacus Software), constraining the E_{max} of each curve to 100%. Drug potency was expressed as the pEC_{50} or pIC_{50} ($-\log EC_{50}$ or $-\log IC_{50}$) for stimulation or inhibition respectively. Non-enzymic [α-^{33}P]-cAMP production (measured at 4°C) was found to be less than 1% of the basal activity (measured at 37°C). The pA_2 for antagonism was determined by Schild analysis of the data where, for a reversible competitive antagonist, provided that the slope is unity, the $pA_2 = pK_B$. The pK_B is $-\log$ of the antagonist equilibrium dissociation constant i.e. $-\log$ (antagonist concentration/(concentration ratio-1)) where concentration ratio is the ratio of the agonist EC_{50} in the absence and presence of antagonist. The antagonist concentrations used were chosen to produce a concentration ratio of between 10 and 100. Data represent the mean$\pm$s.e.m. of at least three separate experiments and all determinations within an experiment were performed in triplicate.

From the *in vivo* duration of action study the relationship between the blood concentration of SB-271046 and MEST response (per cent change in seizure threshold from control) was examined in a direct effect E_{max} pharmacodynamic model. The model produced estimated values for E_{max} (predicted maximal effect at infinite SB-271046 concentration) and EC_{50} (SB-271046 concentration producing 50% of maximal increase in seizure threshold).

Statistical analysis.

Significant differences between drug and vehicle-treated animals on seizure threshold were determined according to the method of Litchfield & Wilcoxon (1949).

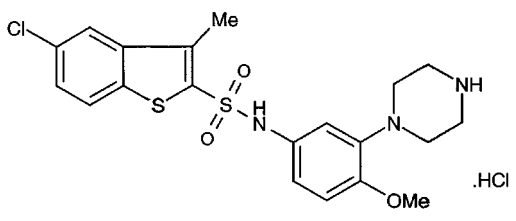

Figure 1 Chemical structure of SB-271046.

Results

Radioligand binding studies

[^{3}H]-LSD and [^{125}I]-SB-258585 binding to recombinant human 5-HT$_6$ receptors displayed a single saturable binding component with a K_d of 1.5 ± 0.1 nM and 0.80 ± 0.05 nM respectively and B_{max} of 3.9 ± 0.8 pmoles mg^{-1} protein and 6.1 ± 0.95 pmoles mg^{-1} protein respectively. In rat and pig stiatum and human caudate putamen tissue, [^{125}I]-SB-258585 again displayed single saturable binding components with K_d of 2.8 ± 0.4, 2.8 ± 0.7, and 1.3 ± 0.04, respectively (see Hirst *et al*., 2000).

SB-271046 displayed high affinity for human recombinant 5-HT$_6$ receptors and 5-HT$_6$ receptors in native brain tissues. At the human cloned receptor, using [^{3}H]-LSD and [^{125}I]-SB-258585 as radioligands, SB-271046 gave pK_is of 8.92 ± 0.04 and 9.09 ± 0.07 respectively (see Table 1.). SB-271046 also potently displaced [^{125}I]-SB-258585 from rat and pig striatal and human caudate putamen membranes with pK_is of 9.02 ± 0.14, 8.55 ± 0.1 and 8.81 ± 0.1 respectively (for inhibition profiles see Hirst *et al*., 2000). SB-271046 had greater than 200 fold selectivity compared to all other 5-HT receptors tested (see Table 1). Moreover, in a comprehensive selectivity screen, 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 (CEREP Task Order 882035).

Adenylyl cyclase measurements

Functional studies on the human cloned 5-HT$_6$ receptor showed a 5-HT concentration-dependent increase in cyclic AMP levels, with a $pEC_{50}=6.74$ (Figure 2a). SB-271046

inhibited the 5-HT stimulation, shifting the 5-HT concentration response curve to the right in a concentration dependent manner with no suppression of the maximal response, consistent with competitive antagonism. Linear regression analysis of Schild plot data (Figure 2b), revealed a correlation coefficient of 0.99, a slope of 1.04 and a $pA_2 = 8.71 \pm 0.3$ for SB-271046, which correlated closely with its 5-HT$_6$ receptor binding affinity. SB-271046 showed no evidence of intrinsic activity in this system.

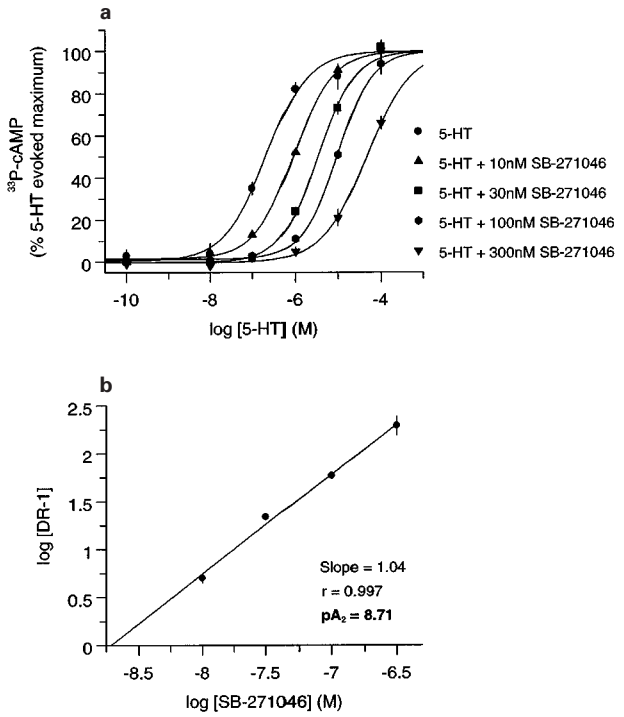

Figure 2 (a) Stimulation of adenylyl cyclase activity in HeLa cells stably expressing human 5-HT$_6$ receptors by 5-HT alone and in the presence of increasing concentrations of SB-271046 (10, 30, 100 and 300 nM). Data points represent the mean and s.e. mean (error bars) of three independent experiments each performed using triplicate determinations. Results are expressed as per cent of the maximal 5-HT response in each assay. (b) Linear regression analysis of this same data displayed as a Schild plot.

Table 1 Selectivity profiles of SB-271046 at recombinant human receptors and native tisue receptors

Receptor	pK_i (mean)
5-HT$_{1A}$	6.35
5-HT$_{1B}$	6.05
5-HT$_{1D}$	6.55
5-HT$_{1E}$	< 4.99
5-HT$_{1F}$	5.95
5-HT$_{2A}$	5.62
5-HT$_{2B}$	5.41
5-HT$_{2C}$	5.73
5-HT$_4$ (guinea-pig hippocampus)	5.27
5-HT$_6$	8.92†
5-HT$_6$	9.09‡
5-HT$_6$ rat striatum	9.02‡
5-HT$_6$ pig striatum	8.55‡
5-HT$_6$ human caudate putamen	8.81‡
5-HT$_7$	5.39
D_2 (long)	5.55
D_3	6.27
Adrenergic α_{1B}	5.76

Data shown are mean values of at least three independent experiments with s.e.mean < 0.2. †pK_i determined with [^{3}H]-LSD, ‡pK_i determined with [^{125}I]-SB-258585. For radioligands for other receptors see Hirst *et al*., 2000.

Maximal electroshock seizure threshold (MEST) test

As shown in Table 2, SB-271046 produced an increase in seizure threshold over a wide-dose range in the rat MEST test, with a minimum significantly effective dose of $\leqslant 0.1$ mg kg^{-1} p.o. At 10 mg kg^{-1} p.o., the compound had a rapid onset of action ($\leqslant 30$ min); reaching a maximum effect at 4 h post-dose (elevating seizure threshold by 166% from a control level of 23.5 ± 2.1 up to 62.5 ± 1.3 mA) and maintaining biological activity for at least 21 h ($P < 0.001$ 30 min – 15 h, $P < 0.05$ 18 – 21 h) (Figure 3). In addition, the level of anticonvulsant activity achieved correlated well with the blood concentrations of SB-271046 observed (Figure 3), with a calculated E_{max} of 155% and EC_{50} of 0.16 μM (Figure 4). Brain samples had low concentrations of SB-271046 with the majority of values below LLQ, although measurable concentrations (0.01 – 0.04 μM, 4.5 – 18.1 ng ml^{-1}) were observed between 2 and 6 h (C_{max}), with the highest levels corresponding to the time of maximum plasma concentration and peak anticonvulsant activity (Figure 3). These determined effective brain levels of SB-271046 are in keeping with its affinity at rat 5-HT6 receptors (pK_i 9.09).

In all studies, the anticonvulsant properties of SB-271046 were evident in the absence of any observed overt behavioural depressant effects (data not shown). Furthermore, even at doses up to 100 mg kg^{-1} p.o., SB-271046 did not impair motor coordination (rotarod test) or spontaneous locomotion (activity counts) in rats (data not shown), suggesting that the anticonvulsant properties of this agent are not secondary to a generalized depressant action.

A time-related increase in seizure threshold was also observed following administration of SB-258510 (10 mg kg^{-1} p.o., 2 – 6 h pre-test). The maximum observed effect (an elevation of seizure threshold of 132% from a baseline of 30 ± 1.2 to 70.8 ± 3.9 mA at 6 h) was comparable to that induced by SB-271046 given at the same dose (Table 2).

Table 2 Effect of SB-271046, SB-258510 and Ro 04-6790 on the threshold for electroshock-induced generalized seizures in rats

Compound	*Dose* (mg kg^{-1})	*Pre-test time* (h)	*Seizure threshold* (mA)
Vehicle	0	4	25.0 ± 1.3
SB-271046	0.1	4	41.5 ± 7.4*
	0.3	4	44.6 ± 3.3***
	1	4	39.2 ± 3.9***
	3	4	44.5 ± 3.7***
	10	4	50.4 ± 7.3***
	30	4	66.7 ± 0.7***
Vehicle	0	2 – 6	30.5 ± 1.2
SB-258510	10	2	48.3 ± 1.7***
	10	4	56.7 ± 1.7***
	10	6	70.8 ± 3.9***
Vehicle	0	1	28.3 ± 1.7
Ro-04-6790	0.3	1	35.5 ± 3.7
	1	1	40.5 ± 2.4***
	3	1	39.5 ± 3.7**
	10	1	41.5 ± 4.7**
	30	1	36.3 ± 2.8*

SB-271046 and SB-258510 were administered orally and Ro 04-6970 was given intraperitoneally. Data represents $EC_{50} \pm$ s.e. values for groups of 12 – 14 rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to corresponding vehicle control group according to Litchfield & Wilcoxon (1949).

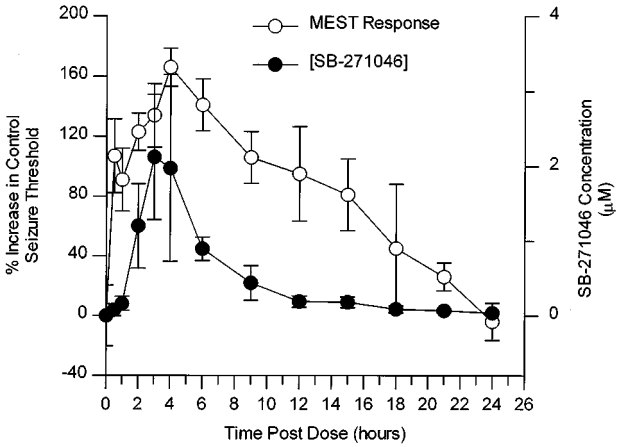

Figure 3 Time course profile of anticonvulsant activity in the MEST test and blood concentration of SB-271046 in rats. SB-271046 was administered at a single dose of 10 mg kg^{-1} p.o. Data represents increase in control $EC_{50} \pm$s.d. values for groups of 11–12 rats. Blood concentrations represent mean $\pm$ s.d. values for groups of five rats.

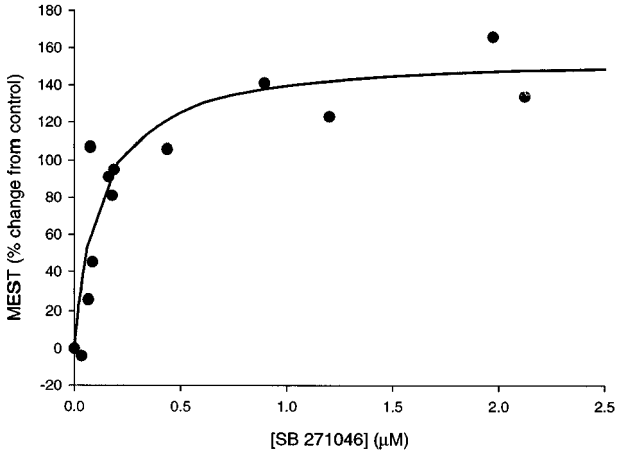

Figure 4 Relationship between SB-271046 blood concentration and seizure threshold. Data represent a direct effect E_{max} pharmacodynamic model plotted using 'WinNonlin Professional Version 1.5' software.

Similarly, Ro 04-6790 produced a modest (maximum of 46%), but significant, elevation of seizure threshold at doses ranging from 1–30 mg kg^{-1} i.p., 1 h pretest (Table 2). The apparently reduced level of anticonvulsant efficacy of Ro 04-6790 compared to SB-271046 and SB-258510, may reflect the poor CNS penetration (<1%) of the former compound (Sleight *et al*., 1998).

Discussion

The present study describes the *in vitro* and *in vivo* characterization of SB-271046, a potent and selective receptor antagonist for the human recombinant 5-HT$_6$ receptor and for rat striatal, pig striatal and human caudate 5-HT$_6$ receptors. The affinities of SB-271046 in rat (pK_i 9.02), pig (pK_i 8.55) and human (pK_i 8.81) were similar suggesting a lack of species differences in this receptor for this given ligand. In addition, this compound has greater than 200 fold selectivity over 69 other receptor, enzyme and binding sites, including all other 5-HT receptor subtypes tested.

In functional studies on human recombinant 5-HT$_6$ receptors, SB-271046 antagonized 5-HT-induced stimulation of adenylyl cyclase activity, in a surmountable manner with a pA_2 of 8.7, which is in good agreement with the radioligand binding affinity of this compound for the 5-HT$_6$ receptor. The inhibition of 5-HT-stimulated adenylyl cyclase activity is competitive, displaying a rightward, concentration-dependent shift of the 5-HT concentration-effect curve with no depression of the maximum response (Figure 2a). Linear regression analysis of Schild plot data revealed a correlation coefficient of 0.99 and a slope of 1.04 (Figure 2b). At concentrations up to 300 nM, SB-271046 displayed no intrinsic activity, having neither stimulatory nor inhibitory activity *per se*. No demonstration of inhibitory activity in the adenylyl cyclase assay suggests that this compound is either a silent antagonist or that there is no constitutive activity of the 5-HT$_6$ receptor in this system, hence no inverse agonism is observed. Failure to demonstrate any partial agonism in this very high receptor expression cell line suggests that this compound is highly unlikely to display intrinsic activity in any native brain tissues.

Following the studies of Schoeffter & Waeber (1994), we have attempted to duplicate these functional studies in rat and pig striatal tissue but we have consistently failed to produce a 5-HT$_6$ receptor mediated stimulation of adenylyl cyclase activity (data not shown).

SB-271046 represents the most selective and potent 5-HT$_6$ receptor antagonist reported to date and therefore, offers significant advantages over other tools/compounds that have been used to characterize the 5-HT$_6$ receptor, both *in vitro* and *in vivo*. For example, whilst SB-271046 has a similar 5-HT$_6$ receptor affinity to those of LSD, a partial agonist at the 5-HT$_6$ receptor, and clozapine and methiothepin, antagonists at the 5-HT$_6$ receptor (Boess *et al*., 1997), it has a superior selectivity profile with respect to other 5-HT receptor subtypes. The identification of Ro 04-6790 (Sleight *et al*., 1998) provided the first selective 5-HT$_6$ receptor ligand. Although the affinity of this compound is relatively low (pK_i 7.4 compared with 8.9 for SB-271046) and the CNS penetration is very limited (<1%), *in vivo* effects of this compound were observed following systemic administration (Bourson *et al*., 1998; Sleight *et al*., 1998).

In the absence of selective 5-HT$_6$ receptor ligands, intracerebroventricular administration of 5-HT$_6$ specific antisense oligonucleotide was used to characterize 5-HT$_6$ receptor function *in vivo*. Whilst this approach was shown to selectively reduce CNS 5-HT$_6$ receptors (using [^{3}H]-LSD as the radioligand for receptor number determination; Bourson *et al*. (1995); Bentley *et al*. (1997); Yoshioka *et al*. (1998)) there are both practical and interpretational difficulties with using this technology *in vivo*. Given these difficulties it is interesting, and perhaps reassuring, to note that there are some similarities between the data obtained using 5-HT$_6$ specific antisense oligonucleotide and selective 5-HT$_6$ receptor antagonists. For example, Bourson *et al*. (1995) showed that administration of 5-HT$_6$ specific antisense oligonucleotide elicited yawning, stretching and chewing in rats, similarly Ro 04-6790 elicited stretching in rats. A similar pattern is seen in animal models of cognition where the same antisense was shown to alter performance in the Morris water maze task (Bentley *et al*., 1997). The latter findings on cognitive function have recently been confirmed and expanded using SB-271046, to include different animal models of cognitive performance (Rogers *et al*., 1999).

The present study shows that SB-271046 produces potent and long-lasting anticonvulsant activity in the rat MEST test, a model of previously reported utility for studying the role of serotonergic pathways in seizure regulation (e.g. Upton *et al*.,

1998). Since SB-271046 is a very selective 5-HT$_6$ receptor antagonist (Bromidge *et al*., 1999; Routledge *et al*., 1999), the anticonvulsant properties of SB-271046 are likely to be mediated by blockade of 5-HT$_6$ receptors. This is further supported by the demonstrated close correlation between the pharmacokinetic and pharmacodynamic profiles of the compound and the presently observed anticonvulsant activity of other selective 5-HT$_6$ receptor antagonists, SB-258510 (Bromidge *et al*., 1999; pK_i at human 5-HT$_6$ receptor 9.2) and the chemically distinct agent Ro 04-6790 (Sleight *et al*., 1998). Overall, these data suggest that the MEST test may provide a robust model of *in vivo* 5-HT$_6$ receptor function, and also illustrate that SB-271046 is a potent and orally active 5-HT$_6$ receptor antagonist. However, the magnitude of these anti-seizure effects was modest in comparison to that of known anti-epileptic drugs For example, using identical test conditions, agents such as carbamazepine can elevate seizure threshold by >1200% (Upton *et al*., 1997) as compared to the maximum increase of only 132 and 166% produced by SB-258510 and SB-271046, respectively. This low level of anticonvulsant efficacy associated with 5-HT$_6$ receptor blockade probably contributes to the apparent lack of dose-dependency for SB-271046, SB-258510 and Ro 04-6790 in the MEST test, since the anticonvulsant activity of SB-271046 is clearly related to the level of exposure in blood. Therefore, the relevance of this observation to the possible clinical utility of SB-271046 in the treatment of epilepsy is, at this stage, unclear.

The anticonvulsant effects of the 5-HT$_6$ receptor antagonists observed in the present studies are intriguing in view of previous evidence indicating that agents which elevate extracellular serotonin, inhibit generalized (and limbic) seizures, whereas agents which deplete brain serotonin are generally associated with proconvulsant activity, as seen in a range of experimental models of epilepsy (see Upton *et al*., 1998).

In addition, blockade of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors with WAY 100635 (0.03–0.3 mg kg^{-1} s.c., 1 h pre-test) (Fletcher *et al*., 1996) and SB-224289 (1–10 mg kg^{-1} p.o., 3 h pre-test) (Gaster *et al*., 1998), respectively, does not alter electroshock-induced seizure threshold in rats (unpublished observations) suggesting that blockade of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors is unlikely to affect seizure propagation. It has also been previously demonstrated that blockade of 5-HT$_3$ receptors (Upton *et al*., 1993) does not induce anticonvulsant activity in the rat intravenous metrazol infusion test. Furthermore, blockade of 5-HT$_{2C}$ and/or 5-HT$_{2B}$ receptors has no effect on electroshock-induced seizure threshold in rats (Upton *et al*., 1998), which is further supported by the lack of anticonvulsant activity of the mixed 5-HT receptor agonist metergoline in this model (unpublished data). It was therefore unexpected that a 5-HT$_6$ receptor antagonist would be anticonvulsant, albeit a modest effect. This suggests that endogenous 5-HT has both anticonvulsant and proconvulsant activity, but under normal physiological conditions, the former dominates.

Taken together, these data demonstrate that SB-271046 is a potent, selective and orally active 5-HT$_6$ receptor antagonist. This compound provides a useful tool for further elucidating the physiological function of 5-HT$_6$ receptors *in vivo*.

We would like to thank Brenda Trail and Tracey Ashmeade for their excellent technical assistance, Phil Jeffrey, Doug Smith, Lisa Bennicosa, Tania Buck, Sarah Murray, and Nigel Deeks for providing pharmacokinetic support and Julia Loudon for her critical evaluation of the manuscript.

References

BENTLEY, J.C., BOURSON, A., BOESS, F.G., FONE, K.C., MARSDEN, C.A., PETIT, N. & SLEIGHT, A.J. (1999). Investigation of stretching behaviour induced by the selective 5-HT$_6$ receptor antagonist, Ro 04-6790, in rats. *Br. J. Pharmacol.,* **126,** 1537–1542.

BENTLEY, J.C., MARSDEN, C.A., SLEIGHT, A.J. & FONE, K.C.F. (1997). 5-HT$_6$ antisense oligonucleotide i.c.v. affects rat performance in the water maze and feeding. British Association for Psychopharmacology Meeting, Cambridge, 13–17th July 1997, P255.

BOESS, F.G., MONSMA, F.J., CAROLO, C., MEYER, V., RUDLER, A., ZWINGELSTEIN, C. & SLEIGHT, A.J. (1997). Functional and radioligand binding characterization of rat 5-HT$_6$ receptors stably expressed in HEK293 cells. *Neuropharmacol.,* **36,** 713–720.

BOURSON, A., BOESS, F.G., BOS, M. & SLEIGHT, A.J. (1998). Involvement of 5-HT$_6$ receptors in nigro-striatal function in rodents. *Br. J. Pharmacol.,* **125,** 1562–1566.

BOURSON, A., BORRONI, E., AUSTIN, R.H., MONSMA, F.J. & SLEIGHT, A.J. (1995). Determination of the role of the 5-ht$_6$ receptor in the rat brain: a study using antisense oligonucleotides. *J. Pharmacol. Exp. Ther.,* **274,** 173–180.

BOWEN, W.P. & JERMAN, J.C. (1995). Nonlinear regression using spreadsheets. *Trends in Pharmacol. Sci.,* **16,** 413–417.

BROMIDGE, S.M., BROWN, A.M., CLARKE, S.E., DODGSON, K., GAGER, T, GRASSAM, H.L., JEFFREY, P.M., JOINER, G.F., KING, F.D., MIDDLEMISS, D.N., MOSS, S.F., NEWMAN, H., RILEY, G., ROUTLEDGE, C. & WYMAN, P. (1999). 5-Chloro-N-(4-methoxy-3-piperazin-1-yl- phenyl)-3-methyl-2-benzothiophenesulfonamide (SB-271046): A Potent, Selective, and Orally Bioavailable 5-ht$_6$ Receptor Antagonist. *J. Med. Chem.,* **42,** 202–205.

CHENG, Y.C. & PRUSSOF, W.H. (1973). Relationship between inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC$_{50}$) of an enzymatic reaction. *Biochem. Pharmacol.,* **92,** 881–894.

FLETCHER, A., FORSTER, E.A., BILL, D.J., BROWN, G., CLIFFE, I.A., HARTLEY, J.E., JONES, D.E., McLENACHAN, A., STANHOPE, K.J., CRITCHLEY, D.J., CHILDS, K.J., MIDDLEFELL, V.C., LANFUMEY, L., CORRADETTI, R., LAPORTE, A.M., GOZLAN, H., HAMON, M. & DOURISH, C.T. (1996). Electrophysiological, biochemical, neurohormonal and behavioural studies with WAY 100635, a potent, selective and silent 5-HT$_{1A}$ receptor antagonist. *Behav. Brain Res.,* **73,** 337–353.

GASTER, L.M., BLANEY, F.E., DAVIES, S., DUCKWORTH, D.M., HAM, P., JENKINS, S., JENNINGS, A.J., JOINER, G.F., KING, F.D., MULHOLLAND, K.R., WYMAN, P.A., HAGAN, J.J., HATCHER, J., JONES, B.J., MIDDLEMISS, D.N., PRICE, G.W., RILEY, G., ROBERTS, C., ROUTLEDGE, C., SELKIRK, J. & SLADE, P.D. (1998). The selective 5-HT$_{1B}$ receptor inverse agonist 1′-methyl-5[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl-2,3,6,7-tetrahydrospiro[furo[2,3-F]indole-3,4′-piperidine] (SB-224289) potently blocks terminal 5-HT autoreceptor function both *in vitro* and *in vivo*. *J. Med. Chem.,* **41,** 1218–1235.

HIRST, W.D., MINTON, J.A.L., BROMIDGE, S.M., MOSS, S.F., LATTER, A.J., RILEY, G., ROUTLEDGE, C., MIDDLEMISS, D.N. & PRICE, G.W. Characterisation of [125I] SB-258585 binding to human recombinant and native 5-HT receptors in rat, pig and human brain tissue. *Br. J. Pharmacol.,* (In Press).

KIMBALL, A.W., BURNETT, W.T. & DOHERTY, D.G. (1957). Chemical protection against ionising radiation I. Sampling methods of screening compounds in radiation protection studies in mice. *Radiation Res.,* **7,** 1–12.

KOHEN, R., METCALF, M.A., KHAN, N., DRUCK, T., HEUBNER, K., LACHOWICZ, J.E., MELTZER, H.Y., SIBLEY, D.R., ROTH, B.L. & HAMBLIN, M.W. (1996). Cloning, characterisation, and chromosomal localisation of a human 5-HT$_6$ serotonin receptor. *J. Neurochem.,* **66,** 47–56.

LITCHFIELD, J.T. & WILCOXON, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.,* **96,** 99–113.

MONSMA, JR. F.J., SHEN, Y., WARD, R.P., HAMBLIN, M.W. & SIBLEY, D.R. (1993). Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol. Pharmacol.,* **43,** 320–327.

ROGERS, D.C., ROBINSON, T.L., QUILTER, C.A., HUNTER, A.J., ROUTLEDGE, C. & HAGAN, J.J. (1999). Cognitive enhancement effects of the selective 5-HT$_6$ antagonist SB-271046. *Br. J. Pharmacol.,* **127,** 22P.

ROTH, B.L., CRAIGO, S.C., CHOUDHARY, M.S., ULUER, A., MONSMA, F.J., SHEN, Y., MELTZER, H.Y. & SIBLEY, D.R. (1994). Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. *J. Pharmacol. Exp. Ther.,* **268,** 1403–1410.

ROUTLEDGE, C, PRICE, G.W., BROMIDGE, S.M., MOSS, S.F., NEWMAN, H., RILEY, G., GAGER, T., BROWN, A.M., LIGHTOWLER, S. & MIDDLEMISS, D.N. (1999). Characterisation of SB-271046, a potent and selective 5-HT$_6$ receptor antagonist. *Br. J. Pharmacol.,* **127,** 21P.

RUAT, M., TRAIFFORT, E., ARRANG, J.M., TARDIVEL-LACOMBE, J., DIAZ, J., LEURS, R. & SCHWARTZ, J.C. (1993). A novel rat serotonin (5-HT$_6$) receptor: molecular cloning, localization and stimulation of cAMP accumulation. *Biochem. Biophys. Res. Comm.,* **193,** 268–276.

SALOMON, Y. (1979). Adenylate cyclase assay. *Adv. Cyclic. Nuc. Res.,* **10,** 35–55.

SCHOEFFTER, P. & WAEBER, C. (1994). 5-Hydroxytryptamine receptors with a 5-HT6 receptor-like profile stimulating adenylyl cyclase activity in pig caudate membranes. *Naunyn Schmiedebergs Arch. Pharmacol.,* **350,** 356–360.

SLEIGHT, A.J., BOESS, F.G., BOS, M., LEVET-TRAFIT, B., RIEMER, C. & BOURSON, A. (1998). Characterization of Ro 04-6790 and Ro 63-0563: potent and selective antagonists at human and rat 5-HT$_6$ receptors. *Br. J. Pharmacol.,* **124,** 556–562.

STEAN, T., ROUTLEDGE, C. & UPTON, N. (1999). Anticonvulsant properties of the selective 5-HT$_6$ receptor antagonist SB-271046-A in the rat maximal electroshock seizure threshold test. *Br. J. Pharmacol.,* **127,** 131P.

UPTON, N., BLACKBURN, T.P., CAMPBELL, C.A., COOPER, D., EVANS, M.L., HERDON, H.J., KING, P.D., RAY, A.M., STEAN, T.O., CHAN, W.N., EVANS, J.M. & THOMPSON, M. (1997). Profile of SB-204269, a mechanistically novel anticonvulsant drug, in rat models of focal and generalised epileptic seizures. *Br. J. Pharmacol.,* **121,** 1679–1686.

UPTON, N., STEAN, T.O. & BLACKBURN, T.P. (1993). BRL 46470A, a selective 5-HT$_3$ receptor antagonist, attenuates the increased seizure susceptibility observed during diazepam-induced withdrawal in mice. *Br. J. Pharmacol.,* **108,** 254P.

UPTON, N., STEAN, T.O., MIDDLEMISS, D., BLACKBURN, T. & KENNETT, G. (1998). Studies on the role of 5-HT$_{2C}$ and 5-HT$_{2B}$ receptors in regulating generalised seizure threshold in rodents. *Eur. J. Pharmacol.,* **359,** 33–40.

YAU, J.L.W., NOBLE, J., WIDDOWSON, J. & SECKL, J.R. (1997). Impact of adrenalectomy on 5-HT6 and 5-HT7 receptor gene expression in the rat hippocampus. *Mol. Brain Res.,* **45,** 182–186.

YOSHIOKA, M., MATSUMOTO, M., TOGASHI, H., MORI, K. & SAITO, H. (1998). Central distribution and function of 5-HT$_6$ receptor subtype in the rat brain. *Life Sci.,* **62,** 1473–1477.

(Received November 5, 1999
Revised March 30, 2000
Accepted May 4, 2000)