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SB-224289 – a novel selective (human) 5-HT_{1B} receptor antagonist with negative intrinsic activity

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1 Human 5-HT_{1B} ($h5\text{-HT}_{1B}$) and human 5-HT_{1D} ($h5\text{-HT}_{1D}$) receptors show remarkably similar pharmacology with few compounds discriminating the receptors. We report here on a novel compound, SB-224289 (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] oxalate), which has high affinity for $h5\text{-HT}_{1B}$ receptors ($pK_I = 8.16 \pm 0.06$) and displays over 75 fold selectivity for the $h5\text{-HT}_{1B}$ receptor over all other 5-HT receptors including the $h5\text{-HT}_{1D}$ receptor and all other receptors tested thus far.

2 Functional activity of SB-224289 was measured in a $[^{35}S]GTP\gamma S$ binding assay on recombinant $h5\text{-HT}_{1B}$ and $h5\text{-HT}_{1D}$ receptors expressed in Chinese Hamster Ovary (CHO) cells. SB-224289 displayed negative intrinsic activity at both receptors with higher potency at $h5\text{-HT}_{1B}$ receptors. SB-224289 caused a rightward shift of agonist concentration response curves consistent with competitive antagonism and generated affinities comparable with those obtained from competition radioligand receptor binding studies.

3 SB-224289 potentiated $[^{3}H]$5-HT release from electrically stimulated guinea-pig cerebral cortical slices to the same extent as as the non-selective 5-HT_1 antagonist methiothepin. SB-224289 also fully reversed the inhibitory effect of exogenously superfused 5-HT on electrically stimulated release.

4 Using SB-224289 as a tool compound, we confirm that in guinea-pig cerebral cortex the terminal 5-HT autoreceptor is of the 5-HT_{1B} subtype.

Keywords: 5-HT; SB-224289; 5-HT_{1B} autoreceptor; inverse agonist

Introduction

The molecular identification of G-protein coupled receptors has greatly outpaced the discovery of compounds to pharmacologically characterize these receptors. The 5-HT receptors are no exception. However, the respite from further novel receptor discovery in this area has recently allowed pharmacology to make significant ground. The family has been divided into seven distinct receptor types, two of which are further subdivided into a number of subtypes with related but distinct amino acid sequences (Hoyer *et al*., 1994). The 5-HT_1 receptor group has been subdivided into 5-HT_{1A} 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} However, until recently only the pharmacology of the 5-HT_{1A} receptor subtype was definitively characterized. The 5-HT_{1B} and 5-HT_{1D} receptors (using the recent nomenclature by Hartig *et al*., 1996) showed remarkably similar pharmacology despite their only modest sequence homology. The 5-HT_{1B} and 5-HT_{1D} subtypes can be pharmacologically identified with the selective $5\text{-HT}_{1B/1D}$ antagonist GR127935 (Skingle *et al*., 1993). However few compounds discriminate between the two receptor subtypes. We have recently published on two compounds, SB-216641 and BRL-15572 (Price *et al*., 1997), which have reasonably high affinity for 5-HT_{1B} and 5-HT_{1D} receptors respectively, with 30 fold and 60 fold selectivity for the respective subtypes. However both GR127935 and SB-216641 are partial agonists at recombinant $h5\text{-HT}_{1B}$ receptors expressed in CHO cell lines, as is BRL-15572 at $h5\text{-HT}_{1D}$ receptors. In native tissues no intrinsic activity has been observed with any of these compounds (Skingle *et al*., 1996; Schlicker *et al*., 1997) and they have been used as antagonists to characterize both serotonergic auto- and hetero-receptors (Roberts *et al*., 1996; Schlicker *et al*., 1997). Nonetheless, if these compounds were used in a system with receptor reserve or highly efficacious receptor-effector coupling, they may display intrinsic activity. We now report on a compound, SB-224289 (Figure 1), which has high affinity and selectivity for 5-HT_{1B} receptors but displays no partial agonism and is an inverse agonist at recombinant $h5\text{-HT}_{1B}$ receptors. A preliminary account of some of the data presented here has been published in an abstract from Selkirk *et al*. (1997a).

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Methods

Receptor expression and membrane preparations

Cloned Receptors Human 5-HT_{1B} and 5-HT_{1D} receptors were stably transfected into the Chinese Hamster Ovary (ACC098) cell line as described by Watson *et al*. (1996). In brief, both cell lines of non-clonal origin were cultured in suspension, in a SmithKline Beecham proprietary medium devoid of serum and nucleosides. Genetecin 418 ($40\ \mu g\ ml^{-1}$) was used as a selection marker for 5-HT_{1D} expressing cells. 5-HT_{1B} receptor expression was amplified by methotrexate [10 μM] selection. Cells were harvested by centrifugation, resuspended in HEPES buffer (20 mM) containing EDTA (10 mM), and homogenized with an Ultra-Turrax. The membranes were then washed in HEPES buffer (20 mM) containing EDTA (0.1 mM), centrifuged and stored as frozen aliquots at $-80^{\circ}C$ until use. These membranes were used in both radioligand binding and $[^{35}S]GTP\gamma S$ binding assays.

SB-224289

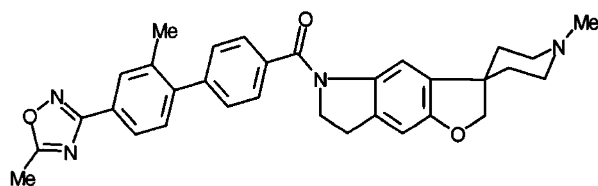

Figure 1 Chemical structure of SB-224289.

Native tissue Guinea-pig cortex was excised and homogenized in Tris buffer (50 mM) containing $MgCl_2$ (10 mM), pH 7.4, using an Ultra-Turrax. The homogenate was incubated at 37°C for 15 min, to degrade endogenous 5-HT. The suspension was then centrifuged at $40{,}000 \times g$ for 10 min, the supernatant discarded and the pellet resuspended in Tris buffer and again centrifuged at $40{,}000 \times g$. This washing procedure was repeated twice more, and the membranes stored at −80°C until use.

Radioligand binding studies

Cloned h5-HT_{1B} and h5-HT_{1D} receptors Membranes were resuspended in Tris buffer (Tris HCl (50 mM), $MgCl_2$ (10 mM), ascorbate (6 mM), pargyline (0.5 μM)), pH 7.4. Competition studies were performed (in single samples) using 10×0.5 log unit dilutions of competing ligand in the presence of [^{3}H]5-HT (4 nM), and non-specific binding was determined by the addition of 5-HT (10 μM). Membranes (10^6 cells) were incubated in a final volume of 0.5 ml for 45 min at 37°C, and the reaction was stopped by rapid filtration through Whatman GF/B filters on a 96-well Filtermate harvester presoaked with 0.3% polyethyleneimine. This was followed by 4×1 ml washes with ice cold Tris buffer. Radioactivity was determined using liquid scintillation counting on a Packard Topcount.

Native tissues Membranes (10 mg original wet weight/well) were resuspended in same Tris buffer as for the cloned receptors and incubated for 30 min at 37°C, in a final volume of 0.5 ml containing [^{3}H]5-CT (1 nM) and 8-OH-DPAT (100 nM) and pizotifen (100 nM) to mask out binding to 5-HT_{1A} receptors and 5-HT_2 receptors respectively. Non-specific binding was determined by the addition of 5-HT (10 μM). The reaction was stopped by rapid filtration through Whatman GF/B filters followed by 5×1 ml washes with ice cold Tris buffer. Radioactivity was determined using liquid scintillation spectrometry.

Selectivity studies To determine the relative receptor selectivities of SB-224289, binding studies were also carried out on cloned human 5-HT_{1A}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_4, 5-HT_6 and 5-HT_7 receptors. Details of cell lines, the ligands and the concentrations used, together with the compounds included to define non-specific binding, are shown in Table 1.

[^{35}S]GTPγS binding studies

CHO cell membranes stably expressing human 5-HT_{1B} or 5-HT_{1D} receptors were prepared as described by Thomas *et al.* (1995), and [^{35}S]GTPγS binding studies were carried out essentially as described by Lazareno *et al.* (1993), with some minor modifications. Briefly, membranes from 10^6 cells were pre-incubated at 30°C for 30 min in 20 mM HEPES buffer (pH 7.4) in the presence of $MgCl_2$ (3 mM), NaCl (100 mM), GDP (10 μM) and ascorbate (0.2 mM), with or without compounds. The reaction was started by the addition of 10 μl of [^{35}S]GTPγS (100 pM, assay concentration) followed by a further 30 min incubation at 30°C. Non-specific binding was determined using nonradiolabelled GTPγS (20 μM) added prior to the membranes. The reaction was terminated by rapid filtration through Whatman GF/B grade filters followed by 5×1 ml washes with ice cold HEPES (20 mM)/$MgCl_2$ (3 mM) buffer. Radioactivity was measured using liquid scintillation spectrometry.

In Vitro [^{3}H]5-HT release studies

Electrically evoked [^{3}H]5-HT release studies were performed as described by Roberts *et al.* (1996), with some slight modifications. Male guinea-pigs (400 – 500 g) were killed by cervical dislocation, decapitated and the brains removed. The whole cortex was rapidly dissected out and cross-chopped into 300 μM $\times$ 300 μM slices using a McIlwain chopper. The slices were incubated with [^{3}H]5-HT (100 nM) in the presence of pargyline (10 μM) at 37°C for 15 min. Slices were then gently washed twice in buffer and resuspended in 10 ml Krebs buffer bubbled with a mixture of 95%O_2/5%CO_2 and 100 μl aliquots transferred to a Brandel Suprafusion 2000 Apparatus. The composition of the Krebs solution was the following (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 10, L-ascorbate 0.06, Na_2EDTA 0.03. Slices were superfused with oxygenated Krebs in the presence

Table 1 Summary of conditions used in binding assays to assess human 5-HT receptor selectivity of SB-224289

Receptor	*Cell line/source*	*Radioligand*	*Concentration*	*NSB (10 μM)*
5-HT_{1A}	HEK 293	[^{3}H]8-OH DPAT	1 nM	Buspirone
5-HT_{1B}	CHO	[^{3}H]5-HT	4 nM	5-HT
5-HT_{1D}	CHO	[^{3}H]5-HT	4 nM	5-HT
5-HT_{1E}	CHO	[^{3}H]5-HT	4 nM	5-HT
5-HT_{1F}	CHO	[^{3}H]5-HT	4 nM	5-HT
5-HT_{2A}	HEK 293	[^{3}H]Ketanserin	0.5 nM	Mianserin
5-HT_{2B}	HEK 293	[^{3}H]5-HT	8 nM	5-HT
5-HT_{2C}	HEK 293	[^{3}H]Mesulergine	0.6 nM	Mianserin
5-HT_4	guinea-pig/piglet hippocampus	[^{125}I]SB-207710	1.3 nM	SB-205008
5-HT_6	HeLa	[^{3}H]LSD	2 nM	Methiothepin
5-HT_7	HEK 293	[^{3}H]5-CT	0.5 nM	5-HT

NSB: compound used to define non-specific binding, all were used at 10 μM.

of the 5-HT uptake blocker paroxetine (10 μM), at a rate of 0.5 ml min^{-1}.

After 32 min of superfusion ($t=0$), samples were collected every 4 min for a period of 80 min. Transmitter release was stimulated electrically with a Brandel Stimulator using a 2 ms biphasic square wave pulse, 20 mA in amplitude. Slices were stimulated at a frequency of 1 Hz for 2 min at $t=-30$ to aid tissue equilibration, and again at $t=12$ (S1) and $t=56$ (S2) at a frequency of 1 Hz for 2 min for reversal of 5-HT inhibition studies, 3 Hz for 1 min to measure the effects on release of the antagonists alone. Agonists were superfused at $t=44$ and removed at $t=64$. Antagonist superfusion was started at $t=24$ and continued for the remainder of the experiment.

At the end of the experiment the amount of radioactivity in the slices and superfusate fractions were determined by liquid scintillation spectrometry. Fractional release (FR) for each sample was calculated as the amount of radioactivity in a sample expressed as a fraction of the total radioactivity present in the slices at the time of that collection. Basal levels of $[^3H]$5-HT release were calculated as the mean percentage FR per 4 min sample of the two samples either side of the S1 and S2 electrical stimulation collection period, and were designated B1 and B2 respectively. S1 and S2 collection periods were for two 4-min samples during and immediately following the electrical stimulation. The effects of drugs on the stimulated release were calculated as (S2-B2) : (S1-B1) ratios of FR, and the results were expressed as this ratio.

Data analysis

Receptor binding data were generated as single data points, and GTPγS data were generated in duplicate within each experiment. Each experiment was carried out at least three times. Curve fitting of the meaned data was generated by a four parameter logistic equation, using GRAFIT (Erithacus Software Ltd). pK_i data were calculated from the IC_{50}, using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). pK_B determinations were made using the Gaddum equation, using concentration-ratios calculated from the EC_{50} from each individual curve.

$[^3H]$5-HT release data were expressed as the S2 : S1 ratio (see above) determined in duplicate within each experiment; data expressed is the mean of $n=3-5$ independent experiments. Statistical significance was determined using analysis of variance *post hoc* Dunnett's *t*-test using the control as standard for the antagonist potentiation studies and the 5-HT [30 nM] as standard for the reversal of inhibition studies.

Materials

SB-224289 (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] oxalate. GR127935 (N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1,-biphenyl]-4-carboxyamide), and Paroxetine HCl were synthesized by SmithKline Beecham Pharmaceuticals (Harlow, U.K.). 5-HT (5-hydroxytryptamine creatine sulphate), GTPγS (Guanosine-5′-*O*-3-thiotriphosphate), GDP (Guanosine 5′-diphosphate, Tris salt) and Pargyline (N-methyl-N-propargylbenzylamine; N-methyl-N-2-propynylbenzylamine) were obtained from Sigma (Poole, U.K.). Methiothepin mesylate was purchased from Research Biochemicals Inc (Natick, MA, U.S.A.). $[^3H]$5-HT (90 Ci $mmol^{-1}$) and $[^{35}S]$GTPγS (900–1200 Ci $mmol^{-1}$) were purchased from Amersham International plc (Buckinghamshire, U.K.).

Results

Radioligand binding studies

Competition studies were performed to determine the affinity of 5-HT, GR127935 and SB-224289 at both $h5\text{-}HT_{1B}$ and $h5\text{-}HT_{1D}$ receptor subtypes. Specific binding with $[^3H]$5-HT (defined with 10 μM 5-HT) represented over 90% of total binding in both $h5\text{-}HT_{1B}$ and $h5\text{-}HT_{1D}$ receptor-expressing systems. Saturation studies (Selkirk *et al*., 1997b) revealed two binding sites in the $h5\text{-}HT_{1B}$ receptor-expressing cell membranes, a high agonist affinity site, $K_D=2.7$ (± 0.8) nM, $B_{max}=3.2$ (± 1.2) pmoles mg^{-1} protein, and a low agonist affinity site, $K_D=239$ (± 219) nM, $B_{max}=38$ (± 22) pmoles mg^{-1} protein (means $\pm$ s.e.mean three independent experiments). At the concentration of $[^3H]$5-HT used in this study (4 nM), negligible binding to this second site would occur. Therefore pK_i data represent binding to the high affinity site only. In the $h5\text{-}HT_{1D}$ receptor-expressing cell membranes $[^3H]$5-HT labels only a single site, $K_D=6.9$ (± 1.1) nM, $B_{max}=8.7$ (± 1.6) pmoles mg^{-1} protein. This K_D was used in the pK_i determinations.

5-HT, GR127935 and SB-224289 displayed high affinity for the $h5\text{-}HT_{1B}$ receptor with the rank order of affinity of GR127935 > 5-HT > SB-224289 (Table 3). The pK_i value for SB-224289 at this receptor is 8.16 ± 0.06, about 75 fold higher than at the human $5\text{-}HT_{1D}$ receptor subtype, $pK_i=6.27 \pm 0.09$, where the rank order of potency for the compounds tested is GR127935 > 5-HT > > SB-224289 (data are means $\pm$ s.e.mean, $n=3-9$). SB-224289 was also equal to or greater than 75 fold selective for the $h5\text{-}HT_{1B}$ receptor over the other human 5-HT receptor subtypes that have been tested (Table 2). SB-224289 also has high affinity at native guinea-pig $5\text{-}HT_{1B}$ receptors, $pK_i=7.91 \pm 0.04$ (Table 2).

$[^{35}S]$GTPγS binding studies

In both $h5\text{-}HT_{1B}$ and $h5\text{-}HT_{1D}$ receptor expressing cell lines, 5-HT produced a concentration-dependent stimulation of $[^{35}S]$GTPγS binding, with a maximal stimulation of 194 and 34% above basal levels respectively (Figure 2a, b). Consistent with its receptor binding affinity, 5-HT had similar potencies at

Table 2 Receptor binding affinities, expressed as pK_i, of SB-224289 at human cloned 5-HT receptors and native tissue

Receptor/Tissue	*pK_i*
$5\text{-}HT_{1A}$	<6
$5\text{-}HT_{1B}$	8.16 ± 0.06
$5\text{-}HT_{1D}$	6.27 ± 0.09
$5\text{-}HT_{1E}$	<5
$5\text{-}HT_{1F}$	<5
$5\text{-}HT_{2A}$	5.92 ± 0.07
$5\text{-}HT_{2B}$	<5.5
$5\text{-}HT_{2C}$	6.2 ± 0.06
$5\text{-}HT_4$	5.69 ± 0.07
$5\text{-}HT_6$	<5.67
$5\text{-}HT_7$	<6
Guinea-pig cortex	7.91 ± 0.04

Data are the means $\pm$ s.e.mean of at least three experiments.

$h5\text{-HT}_{1B}$ and $h5\text{-HT}_{1D}$ receptors (Table 3). Likewise GR127935, a partial agonist at both the $h5\text{-HT}_{1B}$ and $h5\text{-HT}_{1D}$ receptors in this expression system, produced maximal stimulations of 119 and 24% above basal respectively and had similar potency at the two receptor subtypes with potencies in reasonable agreement with the binding affinities (Table 3). SB-224289 however suppressed basal binding by 65% at the $h5\text{-HT}_{1B}$ receptor, and by 32% at the $h5\text{-HT}_{1D}$ receptor. SB-224289 was more potent at $h5\text{-HT}_{1B}$ receptors than $h5\text{-HT}_{1D}$, mirroring the receptor binding affinities. This was clearly a $5\text{-HT}_{1B/1D}$ receptor mediated inhibition of [^{35}S]GTPγS binding since, in cells expressing the $h5\text{-HT}_{1A}$ receptor, SB-224289 produced no effect on [^{35}S]GTPγS binding whereas spiperone was clearly an inverse agonist in this system, consistent with previous findings (Newman-Tancredi *et al*., 1997) (Figure 2c).

Antagonist studies were carried out to determine the potency of SB-224289 at these receptors. At $h5\text{-HT}_{1B}$

Table 3 Receptor binding affinities (pK_i) and [^{35}S]GTPγS binding study results (pEC_{50}, % stimulation, intrinsic activity relative to 5-HT) for 5-HT, GR127935 and SB-224289

	Human $5\text{-}HT_{1B}$ receptors					Human $5\text{-}HT_{1D}$ receptors				
Compound	pK_i	pEC_{50}/ pIC_{50}*	% stimulation	Intrinsic activity	pK_B	pK_I	pEC_{50}/ pIC_{50}*	% stimulation	Intrinsic activity	app. pA_2
5-HT	8.39 ± 0.04	7.82 ± 0.06	194	1.00	–	8.47 ± 0.03	8.24 ± 0.03	34	1.00	–
GR127935	9.03 ± 0.07	8.09 ± 0.10	119	0.61	–	8.60 ± 0.05	8.55 ± 0.10	24	0.71	–
SB-224289	8.16 ± 0.06	7.88 ± 0.07*	−65	–	8.49	6.27 ± 0.09	6.77 ± 0.07*	−32	–	6.94 ± 0.14

All values are expressed as the means ± s.e.mean for five to ten independent experiments (binding studies), four to seven independent experiments ([^{35}S]GTPγS binding studies).

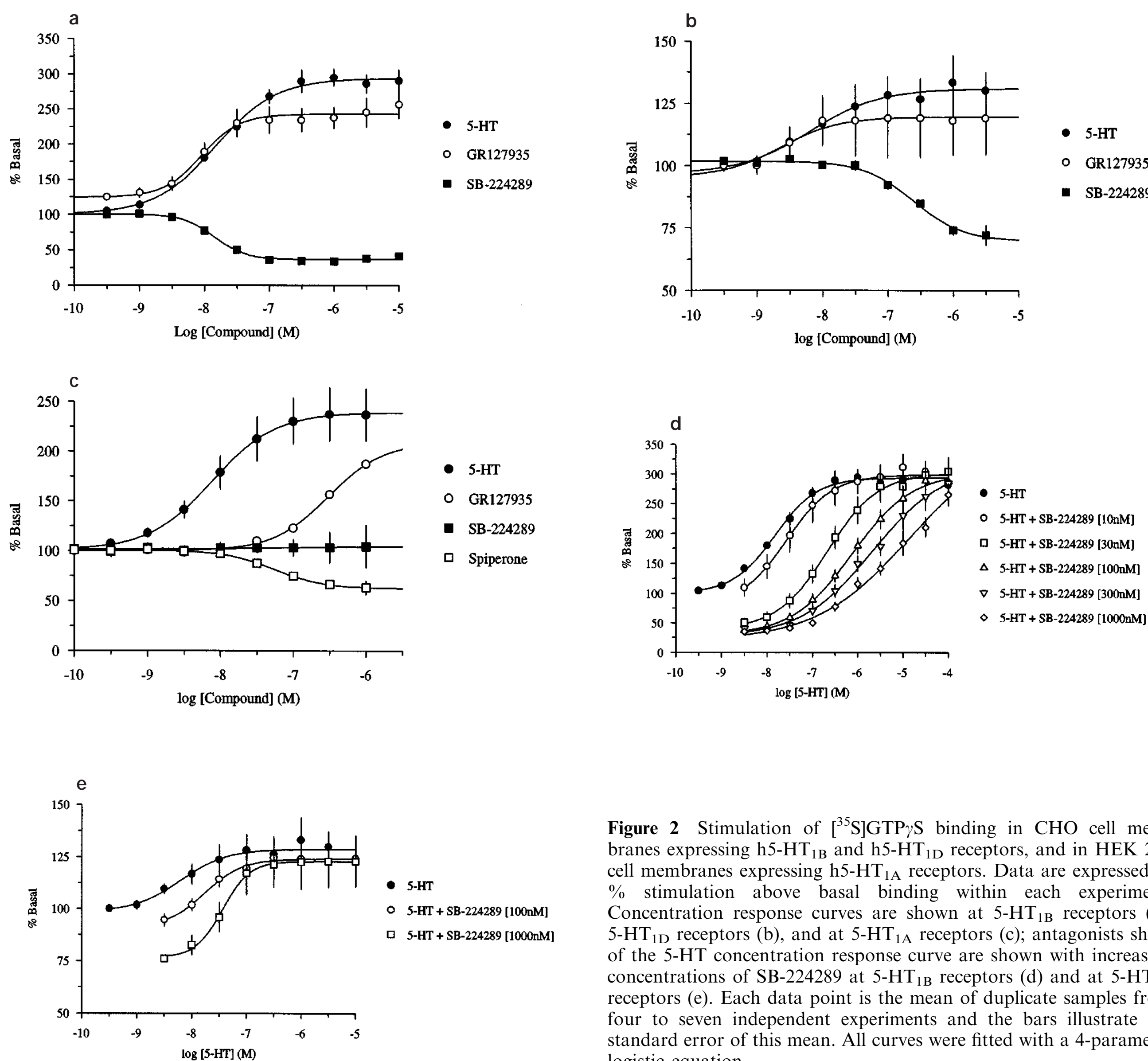

Figure 2 Stimulation of [^{35}S]GTPγS binding in CHO cell membranes expressing $h5\text{-HT}_{1B}$ and $h5\text{-HT}_{1D}$ receptors, and in HEK 293 cell membranes expressing $h5\text{-HT}_{1A}$ receptors. Data are expressed as % stimulation above basal binding within each experiment. Concentration response curves are shown at 5-HT_{1B} receptors (a), 5-HT_{1D} receptors (b), and at 5-HT_{1A} receptors (c); antagonists shifts of the 5-HT concentration response curve are shown with increasing concentrations of SB-224289 at 5-HT_{1B} receptors (d) and at 5-HT_{1D} receptors (e). Each data point is the mean of duplicate samples from four to seven independent experiments and the bars illustrate the standard error of this mean. All curves were fitted with a 4-parameter logistic equation.

receptors, increasing half log concentrations of SB-224289 up to 1 μM produced parallel rightward shifts of the 5-HT concentration response curve with no reduction in the maximal stimulation produced by 5-HT (Figure 2d). This is consistent with competitive antagonism of the 5-HT response. Schild analysis of the data based on pEC_{50}'s of individual curves gave a slope not significantly different from 1 and an apparent pA_2 of 8.49 (data not shown). Likewise in h5-HT_{1D} expressing cells, 100 and 1000 nM SB-224289 produced an apparent pA_2 of 6.94 (Figure 2e). These affinities agree well with those obtained from the radioligand binding studies. All data from the [^{35}S]GTPγS binding studies are summarized in Table 3.

[^{3}H]5-HT release studies

SB-224289 potentiated electrically-evoked (3 Hz, 1 min) [^{3}H]5-HT release from guinea-pig cortical slices in a concentration dependent manner with statistically significant potentiation at 1 μM. This effect is presumably due to SB-224289 antagonism of endogenous 5-HT at the terminal autoreceptor. This effect was also observed with the non selective 5-HT_1 antagonist methiothepin at similar concentrations (Figure 3a).

When slices are stimulated at a lower frequency (1 Hz) the biophase concentration of 5-HT is less. Under these conditions superfusion of 5-HT (30 nM) significantly inhibited electrically stimulated [^{3}H]5-HT release, decreasing the S2 : S1 ratio from 0.87 ± 0.11 to 0.51 ± 0.04 ($P<0.01$). SB-224289 and methiothepin reversed the inhibitory effects of exogenous 5-HT in a concentration dependent manner, statistical significance again being attained by 1 μM ($P<0.05$). This again demonstrates antagonism of the 5-HT terminal autoreceptor by SB-224289. SB-224289 (up to 1 μM) had no effect on basal (that is unstimulated) [^{3}H]5-HT release (data not shown).

Discussion

To date there are few reported compounds which display a high degree of selectivity between h5-HT_{1B} and h5-HT_{1D} receptors. Ketanserin was the first reported compound to display approximately 100 fold selectivity for the h5-HT_{1D} receptor (Kaumann *et al.*, 1994; Zgombick *et al.*, 1995). However its use as a pharmacological tool is restricted due to its relatively low affinity for 5-HT_{1D} receptors together with its very high affinity for other receptors such as 5-HT_{2A} and α_1 adrenoreceptors. Recently Price *et al.* (1997) reported on two novel compounds, SB-216641 and BRL-15572. SB-216641 displays high affinity for h5-HT_{1B} receptors ($pK_1 = 9.0 \pm 0.1$) and 25 fold or greater selectivity over h5-HT_{1D} receptors and all other 5-HT receptor subtypes. BRL-15572 conversely displays higher affinity for h5-HT_{1D} receptors. ($pK_4 = 7.9 \pm 0.1$) and greater than 60 fold selectivity over h5-HT_{1B} receptors. However, SB-216641 is a partial agonist at recombinant h5-HT_{1B} receptors, while BRL-15572, like ketanserin, also has considerable affinity for other receptors, namely h5-HT_{1A} and h5-HT_{2B} receptors and therefore is limited in its use as a pharmacological tool. In the present study we report on the pharmacological properties of SB-224289 which we have shown to be approximately 80 fold selective for the h5-HT_{1B} receptor over all other 5-HT receptor subtypes. Moreover, in a full receptor selectivity screen, SB-224289 was more than 100 fold selective for h5-HT_{1B} receptors over 46 different receptor types and nine enzyme activities (Cerep, study no. 882017), confirming its potential as an excellent pharmacological tool for 5-HT_{1B} receptor subtype characterization.

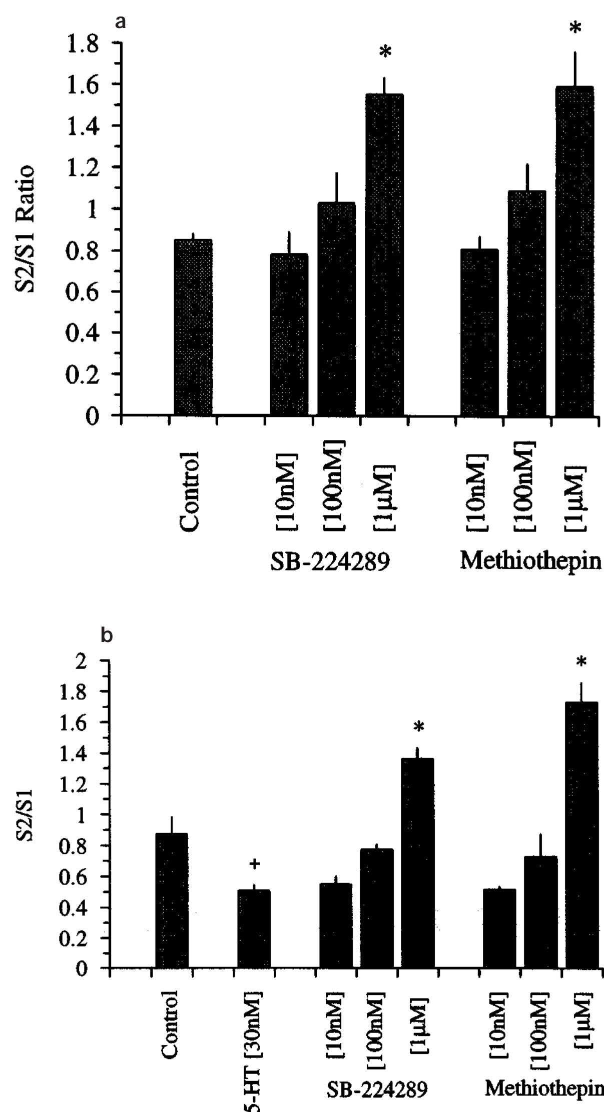

Figure 3 Electrically evoked [^{3}H]5-HT release from guinea-pig cortical slices. Data are shown for the potentiation of [^{3}H]5-HT release by SB-224289 [10, 100, 1000 nM] and Methiothepin [10, 100, 1000 nM] at 3 Hz, 20 mA for 1 min (a), and for the reversal of [^{3}H]5-HT release inhibited by 5-HT [30 nM] by SB-224289 [10, 100, 1000 nM] and Methiothepin [10, 100, 1000 nM] at 1 Hz, 20 mA for 2 min (b). Data is expressed as the mean S2 : S1 ratio for four to six independent experiments and the bars represent the standard error of this mean. $^* = P<0.05$, $+ = P<0.01$.

In the [^{35}S]GTPγS functional assay SB-224289 was compared with the non-selective 5-$HT_{1B/1D}$ antagonist GR127935 in both h5-HT_{1B} and h5-HT_{1D} receptor expressing CHO cell lines. The agonist 5-HT produced a concentration dependent stimulation of [^{35}S]GTPγS binding in both h5-HT_{1B} and h5-HT_{1D} receptor-expressing cells, consistent with agonist stimulation of receptor-G-protein coupling. We confirm that GR127935 showed partial stimulation of basal [^{35}S]GTPγS binding as compared with 5-HT in both h5-HT_{1B} and h5-HT_{1D} receptors, as has been previously reported (Watson *et al.*, 1996: Zgombick *et al.*, 1996), whereas SB-224289 displayed negative intrinsic activity, that is inverse agonism, at both receptors (Figure 2a,b). One possible explanation for the inverse agonism seen in this system is that the G-proteins may be constitutively activated by precoupling to the 5-HT_{1B} and 5-HT_{1D} receptors in the absence of any agonist stimulation (Lefkowitz *et al.*, 1993). However the relevance of inverse

agonism at G-protein coupled receptors has been a subject for debate in the literature and an alternative explanation may be antagonism of an endogenous ligand, that is attenuation of the stimulation resulting from endogenous 5-HT or another endogenous agonist (Baxter & Tilford, 1995). Previous studies in this laboratory have shown that extensive washing of cell membranes however did not alter the drug effects (Thomas *et al*., 1995) and therefore negative efficacy remains the most likely explanation for the elevated basal levels and the inhibitory effects seen on $[^{35}S]GTP\gamma S$ binding with SB-224289. To date though, constitutive coupling of G-proteins has not been convincingly demonstrated in native tissues *in vitro*. It may therefore be a consequence of the artificially high receptor expressing levels seen in recombinant cell systems, although physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor has been demonstrated (Bond *et al*., 1995).

The high expression levels of recombinant receptors also amplify any positive intrinsic activity observed with compounds. GR127935 and SB-216641 display similar degrees of intrinsic activity at cloned h5-HT_{1B} receptors (Watson *et al*., 1996; Zgombick *et al*., 1996; Price *et al*., 1997) yet no intrinsic activity has been observed with either compound in native tissue *in vitro* (Skingle *et al*., 1996: Schlicker *et al*., 1997) or *in vivo* in hypothermia studies (Hagan *et al*., 1997) or in microdialysis studies (Roberts *et al*., 1997). Thus the high intrinsic activity observed may be a consequence of the artificial system. The high degree of receptor reserve present in the h5-HT_{1B} expressing CHO cells has been determined with the non-specific alkylating agent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Watson *et al*., 1996; Zgombick *et al*., 1996). However at the h5-HT_{1D} cloned receptors used in this study, where it has been reported that there is no or little receptor reserve (Watson *et al*., 1996), the same pattern of intrinsic activity is seen suggesting that the partial agonism produced by GR127935 may be reflected at native 5-HT_{1D} receptors. Partial agonism has been reported for GR127935 in the rat neonatal spinal cord thought to contain 5-HT_{1D}-like receptors (Manuel *et al*., 1995). To reiterate, the great advantage of SB-224289 over the existing 5-HT_{1B} receptor antagonists GR127935 and SB-216641 is that this compound exhibits no positive intrinsic activity even in recombinant systems with high receptor reserve. Therefore, where anomalies arise in pharmacological characterization of novel responses using the above compounds, partial agonism can be excluded as a possibility for SB-224289; it can not for GR127935 and SB-216641. In addition, SB-224289 displays nearly 100 fold greater affinity for 5-HT_{1B} receptors over 5-HT_{1D} and all other receptors tested to date. This allows for 90% 5-HT_{1B} receptor blockade with only negligible occupancy of other receptors

In antagonist studies SB-224289 shifted the 5-HT concentration response curve to the right with a pK_B of 8.49 at h5-HT_{1B} receptors upon Schild analysis, and an apparent pA_2 of 6.94 at h5-HT_{1D} receptors where only two concentrations of antagonist were tested. These values agree well with the competition binding affinities and the data are consistent with competitive antagonism.

In the $[^3H]$5-HT release studies SB-224289 acts as an antagonist at the terminal 5-HT autoreceptor strongly suggesting that it is of the 5-HT_{1B} subtype. At higher, frequencies of stimulation SB-224289 caused a significant increase in $[^3H]$5-HT release directly comparable with that caused by the non-selective 5-HT_1 antagonist methiothepin. We attribute this effect to antagonism of endogenous 5-HT rather than inverse agonism (which methiothepin also displays in recombinant h5-HT_{1B} receptor expressing systems (Thomas *et al*., 1995). Studies in synaptosomes where endogenous 5-HT can be removed are required to confirm this. At lower frequencies of stimulation both SB-224289 and methiothepin reversed the inhibition of $[^3H]$5-HT release caused by exogenous 5-HT [30 nM] back to control levels by 100 nM. At 1 μM SB-224289 potentiation of $[^3H]$5-HT above control levels is clearly evident and is due to antagonism of endogenously present 5-HT. At the 1 μM concentration, methiothepin reverses and potentiates $[^3H]$5-HT release to a significantly higher degree than SB-224289. This may be attributed to the non-selective nature of methiothepin acting at heteroreceptors also present at the nerve terminals such as α_2 adrenoreceptors.

In conclusion we have identified SB-224289, a potent compound, selective for the h5-HT_{1B} receptor which, unlike previously identified compounds, displays no positive intrinsic activity at recombinant h5-HT_{1B} receptors expressed in immortal cell lines. It is about 80 fold selective for the h5-HT_{1B} receptor above all other 5-HT receptors and many other receptors and enzymes. SB-224289 is a 5-HT terminal autoreceptor antagonist confirming that this receptor in the guinea-pig brain is of the 5-HT_{1B} subtype.

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