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Characterization of Ro 04-6790 and Ro 63-0563: potent and selective antagonists at human and rat 5-HT$_6$ receptors

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1 This study describes the *in vitro* characterization of two potent and selective 5-HT$_6$ receptor antagonists at the rat and human recombinant 5-HT$_6$ receptor.

2 In binding assays with [^{3}H]-LSD, 4-amino-N-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide (Ro 04-6790) and 4-amino-N-(2,6 bis-methylamino-pyridin-4-yl)-benzene sulphonamide (Ro 63-0563) had mean pK$_i$ values ±s.e.mean at the rat 5-HT$_6$ receptor of 7.35±0.04 and 7.83±0.01, respectively and pK$_i$ values at the human 5-HT$_6$ receptor of 7.26±0.06 and 7.91±0.02, respectively.

3 Both compounds were found to be over 100 fold selective for the 5-HT$_6$ receptor compared to 23 (Ro 04-6790) and 69 (Ro 63-0563) other receptor binding sites.

4 In functional studies, neither compound had any significant effect on basal levels of cyclicAMP accumulation in Hela cells stably expressing the human 5-HT$_6$ receptor, suggesting that the compounds are neither agonists nor inverse agonists at the 5-HT$_6$ receptor. However, both Ro 04-6790 and Ro 63-0563 behaved as competitive antagonists with mean ±s.e.mean pA$_2$ values of 6.75±0.07 and 7.10±0.09, respectively.

5 In rats habituated to observation cages, Ro 04-6790 produced a behavioural syndrome similar to that seen following treatment with antisense oligonucleotides designed to reduce the expression of 5-HT$_6$ receptors. This behavioural syndrome consisted of stretching, yawning and chewing.

6 Ro 04-6790 and Ro 63-0563 represent valuable pharmacological tools for the identification of 5-HT$_6$ receptors in natural tissues and the study of their physiological function.

Keywords: Ro 04-6790; Ro 63-0563; 5-HT$_6$ receptor; 5-HT$_6$ receptor antagonists; stretching

Introduction

The effects of the neurotransmitter 5-hydroxytryptamine (5-HT) are mediated through at least fourteen distinct receptors. The known 5-HT receptors comprise a ligand gated ion channel (the 5-HT$_3$ receptor) and 13 G-protein coupled receptors (Hoyer *et al*., 1994; Boess & Martin, 1994).

The 5-HT$_6$ receptor was first isolated from rat striatal mRNA by reverse transcription and polymerase chain reaction, with degenerate oligonucleotide primers derived from conserved regions of known G-protein coupled receptors (Monsma *et al*., 1993) or by low stringency screening with probes derived from the rat histamine H$_2$ receptor (Ruat *et al*., 1993). Subsequently, the human 5-HT$_6$ receptor has been isolated (Kohen *et al*., 1994). In rats the highest levels of 5-HT$_6$ receptor mRNA are present in olfactory tubercle, nucleus accumbens, striatum and hippocampus (Monsma *et al*., 1993; Ruat *et al*., 1993; Ward *et al*., 1995; Gerard *et al*., 1996). In addition to these regions, 5-HT$_6$ receptor-like immunoreactivity was also identified in frontal and entorhinal cortex and the molecular layer of the cerebellum (Gerard *et al*., 1997).

The 5-HT$_6$ receptor is positively coupled to adenylyl cyclase and can be radiolabelled with [^{125}I]-lysergic acid diethylamide (LSD), [^{3}H]-LSD and [^{3}H]-5-HT (Monsma *et al*., 1993; Boess *et al*., 1997). Many non-selective compounds such as tricyclic antidepressants, antipsychotic agents, tryptamine and ergoline derivatives bind to the 5-HT$_6$ receptor with high affinity (Monsma *et al*., 1993; Roth *et al*., 1994; Boess *et al*., 1997) but to date no selective ligands for the 5-HT$_6$ receptor have been described. Therefore, the identification of functional 5-HT$_6$ receptors in physiological preparations could only be tentative based on the rank order of potency non-selective compounds (Sleight *et al*., 1997).

The only study exploring the functional significance of the receptor *in vivo* used antisense-oligonucleotides which should abolish or reduce the expression of the 5-HT$_6$ receptor protein. Intracerebroventricular treatment of rats with 5-HT$_6$ specific antisense-oligonucleotides produced a behavioural syndrome consisting of yawning, stretching and chewing which could be antagonized by atropine but not by haloperidol (Bourson *et al*., 1995; Sleight *et al*., 1996).

The further study of 5-HT$_6$ receptors and their physiological function requires potent and selective ligands for the receptor. Here we characterize 2,6-dimethylamino-4-sulphanilamido-pyrimidin (Ro 04-6790) and 2,6-dimethylamino-4-sulphanilamido-pyridine (Ro 63-0563) as potent and selective antagonists at the 5-HT$_6$ receptor (Figure 1). These compounds were also used to determine whether 5-HT$_6$ antagonists could produce the same behavioural syndrome as that induced by treatment with antisense oligonucleotides *in vivo*.

Methods

Animals

Male Sprague-Dawley rats (Füllinsdorf, Switzerland), weighing 250–300 g, were used for the experiment. They were housed in groups of 4 under controlled conditions of temperature (21±1°C) and humidity (55–65%) and under a

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12 h light-dark cycle. Rats were allowed free access to food and water.

Receptor binding assays

The affinities of Ro 04-6790 and Ro 63-0563 at rat and human 5-HT$_6$ receptors were measured on membranes obtained from HEK 293 and Hela cells stably expressing the rat and human receptors, respectively. HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) +10% foetal bovine serum (FBS) containing penicillin (100 iu ml^{-1}), streptomycin (100 μg ml^{-1}) and 0.5 mg ml^{-1} geneticin in a humidified atmosphere (5% CO_2). Hela cells were grown in exactly the same conditions except that geneticin was omitted from the media. The cells were detached with phosphate buffered saline (PBS) containing 1 mM EDTA, washed with PBS by two centrifugations (10 min, 500 *g*) and the resulting pellet was resuspended in 50 mM, ice-cold Tris-HCl (pH 7.4), containing 10 mM $MgCl_2$ and 0.5 mM EDTA by use of a polytron homogenizer (15 sec at maximal speed), at a concentration corresponding to 4×10^7 cells ml^{-1} and aliquots were stored at -80°C. 5-HT$_6$ receptor binding assays were performed with [^{3}H]-lysergic acid diethylamide ([^{3}H]-LSD; specific activity 86 Ci mmol^{-1}, Amersham). Membranes corresponding to 4×10^5 cells/assay tube were used for the binding assay, resuspended in an assay buffer consisting of Tris-HCl 50 mM, pargyline 10^{-5} M, $MgCl_2$ 5 mM and ascorbic acid 0.1%, pH 7.4. For estimations of the expression levels and the affinity of [^{3}H]-LSD for the receptor binding sites, saturation experiments were performed with 8 concentrations of [^{3}H]-LSD (0.163–20 nM). Competition curves were constructed with 7 concentrations of the displacing agents (1 data point per log unit of concentration: 10^{-10} M to 10^{-4} M). Binding assays consisted of 100 μl of the membrane preparation expressing the 5-HT$_6$ receptor, 50 μl of [^{3}H]-LSD and 50 μl of a displacing drug or assay buffer. Non-specific binding was measured in the presence of 10^{-5} M 5-HT. Incubations were carried out for 1 h at 37°C and reactions were stopped by rapid filtration through Whatmann GF/B filters by use of a Filtermate 196 (Packard Canberra). The filters were washed with 3×2 ml Tris HCl (50 mM, pH 7.4) and the radioactivity retained on the filters was measured by scintillation spectroscopy in 50 μl of scintillation fluid. All experiments were performed in triplicate and repeated 3 times. The dissociation constants for [^{3}H]-LSD binding to both human and rat 5-HT$_6$ receptors, IC_{50} values, K_i values and Hill coefficients were calculated by use of EBDA and LIGAND (Munson & Rodbard, 1980; McPherson, 1985).

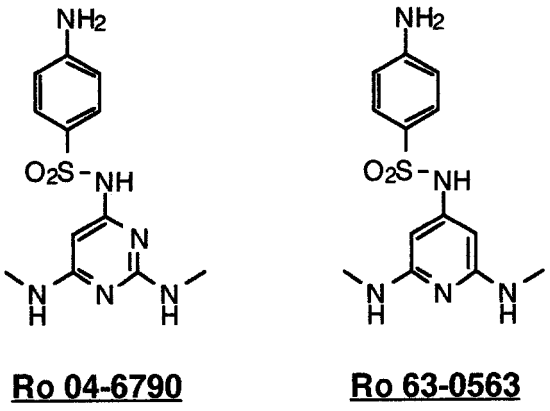

Figure 1 Chemical structures of Ro 04-6790 and Ro 63-0563.

In all other receptor binding assays, Ro 04-6790 and Ro 63-0563 were tested at a single concentration (10^{-5} M) by the methodology described in Table 1. If either compound displaced more than 50% of the specific binding in any of these assays, it was retested at multiple concentrations to estimate pK_i values. Ro 63-0563 was also tested in a further 45 binding assays by CEREP (Le Bois l'Eveque, 86600 Celle L'Evescault, France). Here the compound was tested at concentrations of 0.1 and 10 μM at the adenosine receptor subtypes 1 and 2 (rat) and 3 (human); angiotensin receptor subtypes 1 (rat) and 2 (bovine); bradykinin receptor subtypes 1 (rat) and 2 (human); calcitonin gene-related peptide receptors; cholecystokinin receptor subtypes A and B (human); endothelin receptor subtypes A and B (human); $GABA_A$ and $GABA_B$ receptor subtypes (rat); galanin receptors; glycine receptors (both strychnine sensitive and insensitive); histamine receptor subtypes H_1 (CNS, guinea-pig) and H_1 (peripheral, guinea-pig), H_2 (guinea pig) and H_3 (rat); melatonin receptors (chicken); neuropeptide Y receptor 1 and 2 subtypes; neurokinin receptor subtypes 1, 2 and 3 (human); neurotensin receptors; P2X and P2Y purinoceptors (rat); somatostatin receptors. Ro 63-0563 (0.1 μM and 10 μM) was also tested at adenosine (guinea-pig), noradrenaline (rat), dopamine (rat) and 5-HT (rat) uptake sites and at various ion channels, including several subtypes of calcium channels (L, DHP site, L, ditiazem site, L, verapamil site and N), potassium channels (ATP-sensitive, voltage-dependent and Ca^{2+}-dependent) and sodium channels.

Adenylyl cyclase measurements

Hela cells expressing human recombinant 5-HT$_6$ receptors were grown to 90% confluency in DMEM +10% FBS (dialyzed), washed once with DMEM without phenol red (DMEM−), detached with PBS +1 mM EDTA and washed 2× with DMEM (450 *g*, 5 min). The final cell density was adjusted to approximately 1.25×10^6 cells ml^{-1}. Aliquots of 80 μl were transferred to 96 well plates (approximately 10^5 cells/well) and incubated at 37°C in a humidified atmosphere for 30 min. 5-HT, combined with pargyline and the phosphodiesterase inhibitor Ro 20-1724, was added in a volume of 20 μl/well (final incubation volume 100 μl/well, final concentration of pargyline and Ro 20-1724: 20 μM and 100 μM, respectively). In order to determine the agonist potential of the two compounds, full concentration-response curves were constructed with Ro 04-6790 and Ro 63-0563 at the h5-HT$_6$ receptor and compared to 5-HT as a positive control. To determine the antagonist affinities of the 2 compounds full dose-response curves to 5-HT were constructed in the presence of either 1, 3, 10 and 30 μM Ro 04-6790 or 0.1, 0.3, 1 and 3 μM Ro 63-0563. After a period of 20 min at 37°C in a humidified atmosphere (5% CO_2), the incubation was terminated by the addition of 200 μl ethanol/well. All experiments were performed in triplicate and repeated at least 3 times. After at least 2 h at -20°C, the plates were centrifuged for 5 min at 470 *g* (4°C) and 75 μl aliquots of the supernatant were transferred to Packard OptiPlates, evaporated under vacuum, and resuspended in 0.05 M acetate buffer. The concentration of adenosine 3′:5′-cyclic monophosphate (cyclicAMP) was determined by use of the BIOTRAK cyclicAMP [^{125}I] scintillation proximity assay (SPA) system (Amersham) adapted to 96-well plates. Agonist dose-response curves were analysed from the equation; $E = B + E_{max} \times x/(EC_{50} + x)$, where x is the concentration of agonist, E and E_{max} the measured and the maximum effect (cyclicAMP/well), EC_{50} is

Table 1 Methodologies for the binding assays used to determine the selectivity of Ro 04-6790 and R0 63-0563

Receptor	*Tissue/expression system*	*Radioligand (concentration)*	*Non-specific ligand (concentration)*	*Incubation conditions*
h5-HT$_{1A}$	Human recombinant /3T3 cells	[^{3}H]-5-HT (1 nM)	5-HT (10 μM)	60 min: Room temp.
5-HT$_{1B}$	Rat striatum	[^{3}H]-5-HT (1 nM) 8-OH-DPAT (300 nM) Mesulergine (300 nM)	5-HT (10 μM)	60 min: Room temp.
h5-HT$_{1D}$	Human recombinant /HEK 293 cells	[^{3}H]-LSD (2 nM)	5-HT (10 μM)	60 min: Room temp.
h5-HT$_{2A}$	Human recombinant /3T3 cells	[^{3}H]-DOB (1 nM)	Methysergide (10 μM)	60 min: Room temp.
h5-HT$_{2C}$	Human recombinant /3T3 cells	[^{3}H]-5-HT (1 nM)	5HT (10 μM	60 min: Room temp.
5-HT$_3$	N18 cells	[^{3}H]-GR 65630 (0.2 nM)	Metoclopramide (10 μM)	60 min: 4°C
5-HT$_4$	Guinea-pig striatum	[^{3}H]-GR 113808 (0.1 nM)	5-HT (30 μM)	30 min: 37°C
h5-HT$_7$	Human recombinant /CHO cells	[^{3}H]-LSD (2 nM)	Methiothepin (10 μM)	60 min: 37°C
hD$_1$	Human recombinant /GH4 cells	[^{3}H]-SCH 23390 (0.5 nM)	(+)-Butaclamol (10 μM)	45 min: Room temp.
hD$_2$	Human recombinant /CHO cells	[^{3}H]-spiperone (1.6 nM)	(+)-Butaclamol (10 μM)	60 min: Room temp.
hD$_3$	Human recombinant /CHO cells	[^{3}H]-YM-09151-2 (0.1 nM)	(+)-Spiperone (10 μM)	60 min: Room temp.
hD$_4$	Human recombinant /CHO cells	[^{3}H]-spiperone (1.6 nM)	(+)-Butaclamol (10 μM)	60 min: Room temp.
hD$_5$	Human recombinant /CHO cells	[^{3}H]-SCH 23390 (1 nM)	(+)-Butaclamol (10 μM)	60 min: Room temp.
α_1-Adrenoceptor	Rat cortex	[^{3}H]-prazocin (1 nM)	Phentolamine (10 μM)	60 min: Room temp.
α_2-Adrenoceptor	Rat cortex	[^{3}H]-clonidine (1 nM)	Yohimbine (10 μM)	60 min: Room temp.
β-Adrenoceptor	Rat cortex	[^{3}H]-DHA (0.2 nM)	Propranolol (10 μM)	60 min: Room temp.
hM$_1$	Human recombinant /CHO cells	[^{3}H]-pirenzepine (2 nM)	Atropine (1 μM)	60 min: Room temp.
hM$_2$	Human recombinant /CHO cells	[^{3}H]-AF-DX 384 (3 nM)	Atropine (1 μM)	60 min: Room temp.
hM$_3$	Human recombinant /CHO cells	[^{3}H]-4-DAMP (0.2 nM)	Atropine (1 μM)	60 min: Room temp.
hM$_4$	Human recombinant /CHO	[^{3}H]-4-DAMP (0.2 nM)	Atropine (1 μM)	60 min: Room temp.
hM$_5$	Human recombinant /CHO	[^{3}H]-4-DAMP (0.2 nM)	Atropine (1 μM)	60 min: Room temp.
μ-Opioid	Rat recombinant /CHO	[^{3}H]-naloxone (3 nM)	Naloxone (10 μM)	30 min: Room temp.
κ-Opioid	Rat recombinant /CHO	[^{3}H]-naloxone	Naloxone (10 μM)	30 min: Room temp.
Benzodiazepine	Rat cortex	[^{3}H]-flumazenil (1 nM)	Diazepam (10 μM)	30 min: 4°C

The compounds were tested at a single concentration of 10^{-5} M and if more than 50% of the specific binding was displaced at this concentration, full displacement curves could be constructed to determine pK_i values.

the concentration of agonist producing 50% of the response and B the basal cyclicAMP level. pA_2 values were calculated as described by Arunlakshana & Schild (1959) by calculating the ratio of the EC_{50} for 5-HT in the presence or absence of various concentrations of each antagonist. A pA_2 value was calculated for each separate experiment together with the gradient of the Schild regression. The experiment was performed 3 times and the mean pA_2 values $\pm$s.e.mean and mean slopes $\pm$s.e.mean were calculated for both Ro 04-6790 and Ro 63-0563.

Measurement of Ro 04-6790 and Ro 63-0563 in CSF and plasma

Rats were injected with Ro 04-6790 (30 mg kg^{-1}, i.p.) or Ro 63-0563 (10 mg kg^{-1}, i.v.) and 0.5, 1, 2, 3 and 6 h later the rats were anaesthetized with 5% isoflurane 95% O_2. CSF samples were taken from the cisterna magna by inserting a 22 G needle between the 1st and 2nd cervical vertebra. A sample of blood was then removed from the aorta and stored in tubes each containing 7 μg EDTA and 5 μg NaF to prevent coagulation. Blood and CSF samples were taken from 3 rats 0.5 h following administration of the 5-HT$_6$ antagonists, from 2 rats 1 h after administration and from 1 rat at 2, 3 and 6 h after. Blood samples were centrifuged for 5 min at 1200 r.p.m. and CSF and plasma samples were stored at -20°C until analysis.

Samples were analysed by high performance liquid chromatography with ultra violet detection (h.p.l.c.-u.v.) with gradient elution and a 150×4 mm analytical column packed with Inertsil ODS-3.5 μm (GL Sciences Inc. U.S.A.). The two mobile phases contained a mixture of acetonitrile and 50 mM $NaHPO_4$ (pH 5.5). U.V. detection was performed at 290 nm for Ro 04-6790 and 270 nm for Ro 63-0563. Plasma samples of 100 μl were deproteinated by adding 100 μl $HClO_4$ (1 N). Following centrifugation for 5 min at 1200 r.p.m., 100 μl of the supernatant were injected onto the column. CSF samples (50 μl) were diluted with 50 μl H_2O and injected directly onto the column. The limits of detection of Ro 04-6790 in CSF and plasma were 6 ng ml^{-1} and 80 ng ml^{-1}, respectively, and those for Ro 63-0563 were 8 ng ml^{-1} and 80 ng ml^{-1}, respectively.

Behavioural observations

Every day for 4 consecutive days, rats were injected intraperitoneally with saline (1 ml kg^{-1}) and immediately placed in transparent plexiglass cages (55 × 34 × 18 cm) in groups of 4 for 1 h. A mirror was positioned behind the cages to allow all-round observation of the rats. On the 5th day, rats received either Ro 04-6790 (3, 10, 30 mg kg^{-1}, i.p.) or saline and were again immediately placed in the observation boxes (4 groups of 4 per cage) into which they had been habituated with one animal per treatment group in each box. The number of yawns and stretches was counted by direct observation for a period of 60 min from the time that the rats were treated with the 5-HT$_6$ antagonist. The experiments were performed in this manner so that the results obtained could be compared with those obtained in animals treated with antisense oligonucleotides designed to block the translation of 5-HT$_6$ receptors (Bourson *et al*., 1995). In these published experiments, the animals were injected daily with the oligonucleotides and then placed in observation cages each day. These experiments were carried out between 8 h 30 min and 12 h 30 min; all behavioural studies were performed on a 'blind' basis.

Analysis of behavioural data

The results are expressed as the mean number of yawns or stretches per hour per group. Although these data were not normally distributed, in Figure 7, s.e.mean values are also given for an indication of variability. A Kruskall-Wallis analysis of variance was first carried out, and if significant, a Mann-Whitney *U* test was used to compare differences between treatment groups.

Materials

[^{3}H]-5-HT, [^{3}H]-LSD, [^{3}H]-GR 113808 ([1[2-(methylsuphonyl)-aminoethyl] - 4 - piperidinyl]methyl - 1-methyl-1H-indole-3-carboxylate), [^{3}H]-SCH 23390 (7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride), [^{3}H]-spiperone, [^{3}H]-prazocin, [^{3}H]-DHA (dihydroalprenolol) and [^{3}H]-flumazenil were purchased from Amersham (U.K.) and [^{3}H]-DOB ([^{3}H]4-bromo-2,5-dimethoxyphenylisopropylamine), [^{3}H]-GR 65630 ([^{3}H]3-(5-methyl-1*H*-imidazol-4-yl)-1-(1 - methyl - 1*H* - indol - 3 - yl) - 1 - propanone), [^{3}H] - YM-09151-2 ([^{3}H](±)-cis-N-1-benyl-2-methylpyrrolidin-3-yl)-5-chloro-2-methyoxy-4-methylamiono benzamide), [^{3}H]-clonidine, [^{3}H]-pirenzepine, [^{3}H]-AF-DX 384 ([^{3}H](±)-5,11-dihydro-11-[2-2-[(dipropylamino)methyl] - 1 - piperidinylethylamino]carbonyl-6H - pyrido(2,3-b)(1,4) - benzodiazepine - 6 - one), [^{3}H] - DAMP and [^{3}H]-naloxone were purchased from New England Nuclear, (U.S.A.). 5-HT was purchased from Fluka (Switzerland). Atropine, (+)-butaclamol, metoclopramide, methiothepin, methysergide, naloxone, pargyline phentolamine, propranolol, spiperone and yohimbine were purchased from Research Biochemicals International U.S.A. DMEM, FBS, penicillin, streptomycin and geneticin were obtained from GIBCO Life Technologies and Ro 20-1724 (4-(3-butoxy-4-methylbenzyl)-2-inidazolidinone), Ro 04-6790, Ro 63-0563 and diazepam were synthesized at F. Hoffmann-La Roche.

Results

[^{3}H]-LSD labelled recombinant rat 5-HT$_6$ receptors with a dissociation constant (K_d) of 1.9 ± 0.1 nM and recombinant human 5-HT$_6$ receptors with a K_d of 1.6 ± 0.1 nM. As can be seen in Figure 2, both Ro 04-6790 and Ro 63-0563 displaced [^{3}H]-LSD from both the human and the rat 5-HT$_6$ receptor with high affinity. The affinity values (mean ± s.e.mean pK$_i$) of Ro 04-6790 for the rat and human 5-HT$_6$ receptor were 7.35 ± 0.04 and 7.26 ± 0.06, respectively (Table 2). Ro 63-0563 had higher affinity than Ro 04-6790 for the rat and human 5-HT$_6$ receptors with pK$_i$ values of 7.83 ± 0.01 and 7.91 ± 0.02, respectively (Table 2). The curves shown in Figure 2 represent the means of 3 experiments for both compounds.

Ro 04-6790 has over 100 fold selectivity for the 5-HT$_6$ receptor with respect to the other receptor binding sites examined. We were unable to measure the affinity of Ro 04-6790 for any of the other 23 receptor binding sites studied

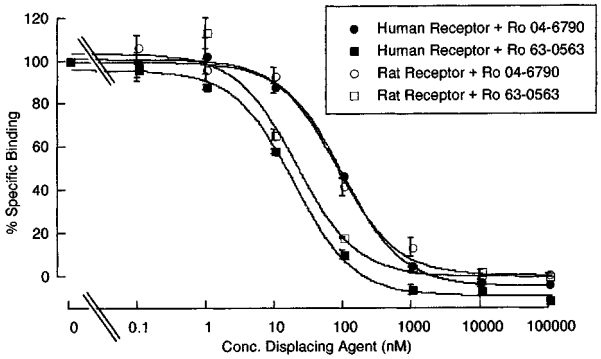

Figure 2 Displacement of [^{3}H]-LSD from rat recombinant 5-HT$_6$ receptors by Ro 04-6790 and Ro 63-0563 and from human recombinant 5-HT$_6$ receptors by Ro 04-6790 and Ro 63-0563. Each data point represents the mean of 3 separate competition experiments; vertical lines show s.e.mean.

Table 2 Affinities of Ro 04-6790 and Ro 63-0563 for various binding sites

Receptor	*Ro 04-6790*	*Ro 63-0563*
h5-HT$_6$	7.26 ± 0.06	7.91 ± 0.02
r5-HT$_6$	7.35 ± 0.04	7.83 ± 0.01
h5-HT$_{1A}$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
5-HT$_{1B}$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
h5-HT$_{1D}$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
h5-HT$_{2A}$	IC$_{50}$ > 10 μM	5.32 ± 0.02
h5-HT$_{2C}$	IC$_{50}$ > 10 μM	5.69 ± 0.01
5-HT$_3$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
5-HT$_4$	ND	IC$_{50}$ > 10 μM
h5-HT$_7$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hD$_1$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hD$_2$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hD$_3$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hD$_4$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hD$_5$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
α_1-Adrenoceptor	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
α_2-Adrenoceptor	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
β-Adrenoceptor	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hM$_1$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hM$_2$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hM$_3$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hM$_4$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
hM$_5$	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
r μ-Opioid	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
r κ-Opioid	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM
Benzodiazepine	IC$_{50}$ > 10 μM	IC$_{50}$ > 10 μM

Affinities are given as pK$_i$ values except where 10 μM of the displacing agent failed to displace 50% of the specific binding. ND = not determined.

(Table 2), since it failed to displace more than 50% of the specific binding at a concentration of 10 μM. Indeed, for all of the other 5-HT receptors tested there was no displacement of specific binding by 10 μM Ro 04-6790.

Ro 63-0563 also had over 100 fold selectivity for the 5-HT$_6$ receptor with respect to the other binding sites tested. The only other receptors for which Ro 63-0563 had any measurable affinity were the 5-HT$_{2A}$ and the 5-HT$_{2C}$ receptors with pK$_i$ values of 5.32 ± 0.02 and 5.69 ± 0.01, respectively (Table 2). In all other binding assays, Ro 63-0563 failed to displace more than 50% of the specific binding at a concentration of 10 μM. This was also true for all the binding sites tested at CEREP (see Methods section).

At the human 5-HT$_6$ receptor, neither Ro 04-6790 nor Ro 63-0563 had any agonist activity (Figure 3), since they had no effect on the accumulation of cyclicAMP. In addition no inverse agonist activity was seen in that the compounds themselves did not significantly reduce basal accumulation of cyclicAMP (Figure 3). However, both Ro 04-6790 and Ro 63-0563 behaved as competitive antagonists. Ro 04-6790 had a pA$_2$ value of 6.75 ± 0.07 calculated from Schild regressions with a slope of 1.16 ± 0.04. The pA$_2$ value of Ro 63-0563 at the 5-HT$_6$ receptor was 7.10 ± 0.09 calculated from Schild regressions with a slope of 1.14 ± 0.13. All of these values are means $\pm$ s.e.mean of 3 separate experiments each with 4 concentrations of each antagonist. Figure 4 shows a typical example of concentration-response curves to 5-HT in the presence of different concentrations of Ro 04-6790 and, likewise, Figure 5 shows a typical example of concentration-response curves to 5-HT in the presence of different concentrations of Ro 63-0563.

The measurement of Ro 04-6790 and Ro 63-0563 in both plasma and cerebrospinal fluid (CSF) showed that a small but significant fraction of the plasma concentration of Ro 04-6790 could be detected in the CSF (Figure 6). After treatment with Ro 04-6790 (30 mg kg^{-1}, i.p.), its concentration in the CSF was sufficient to occupy more than 70% of the 5-HT$_6$ receptors. Ro 63-0563 was detected in plasma at similar concentrations to Ro 04-6790 but could not be detected in the CSF at any time point (results not shown). Therefore, Ro 04-6790 alone was used for behavioural studies, to determine whether the same behavioural syndrome could be produced as in rats treated with antisense oligonucleotides designed to prevent the translation of 5-HT$_6$ mRNA. Animals were habituated to the observation cages for 4 days. On the 5th day, rats treated with Ro 04-6790 exhibited a behavioural syndrome of yawning, stretching and chewing identical to that produced by antisense oligonucleotides. As can be seen from Figure 7, Ro 04-6790 produced a dose-related and statistically significant increase in the number of stretches observed over a 1 h period immediately after treatment. A maximal effect was observed at a dose of 30 mg kg^{-1}, i.p., which is in agreement with the concentration of Ro 04-6790 in the CSF being sufficient to occupy 5-HT$_6$ receptors. Similar results were observed for yawning. However, this failed to reach significance ($P = 0.17$, data not shown).

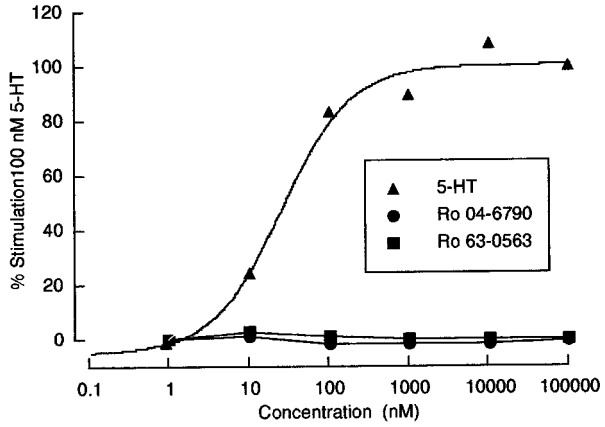

Figure 3 Effect of 5-HT, Ro 04-6790 and Ro 63-0563 on cyclicAMP accumulation in Hela cells stably expressing the recombinant human 5-HT$_6$ receptor. Data points represent the means of at least 3 separate experiments.

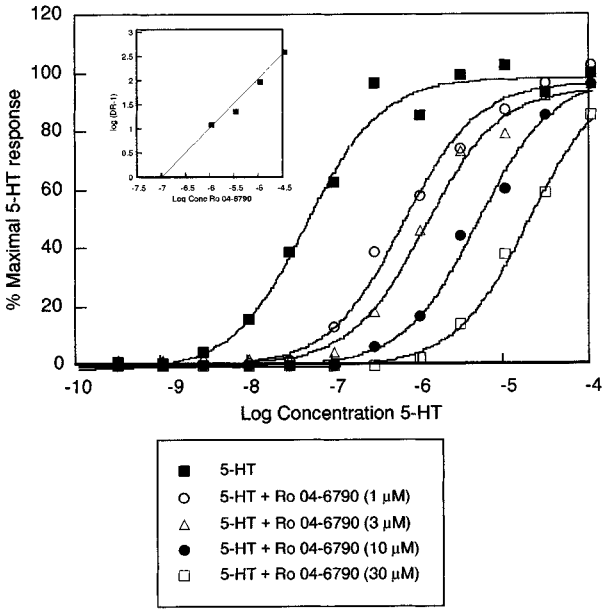

Figure 4 Stimulation of cyclicAMP accumulation in Hela cells stably expressing the human 5-HT$_6$ receptor by either 5-HT alone or 5-HT in the presence of Ro 04-6790 (1, 3, 10 or 30 μM). This figure shows a typical example of 3 separate experiments. Inset, Schild regression of the data.

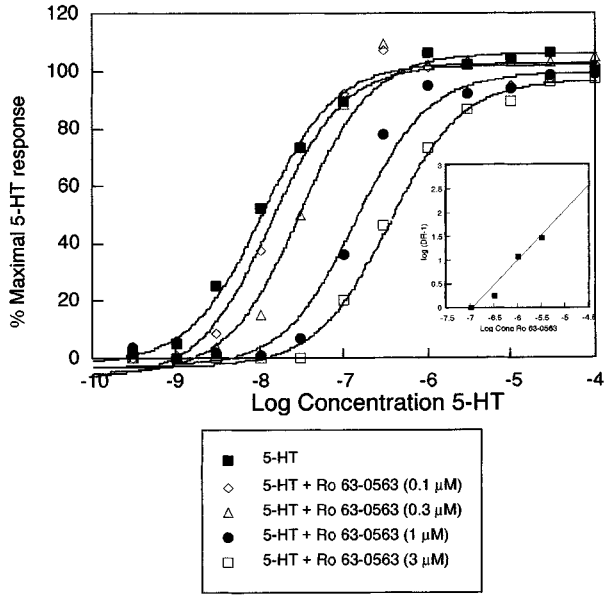

Figure 5 Stimulation of cyclicAMP accumulation in Hela cells stably expressing the human 5-HT$_6$ receptor by either 5-HT alone or 5-HT in the presence of Ro 63-0563 (0.1, 0.3, 1 or 3 μM). This figure shows a typical example of 3 separate experiments. Inset, Schild analysis of the data.

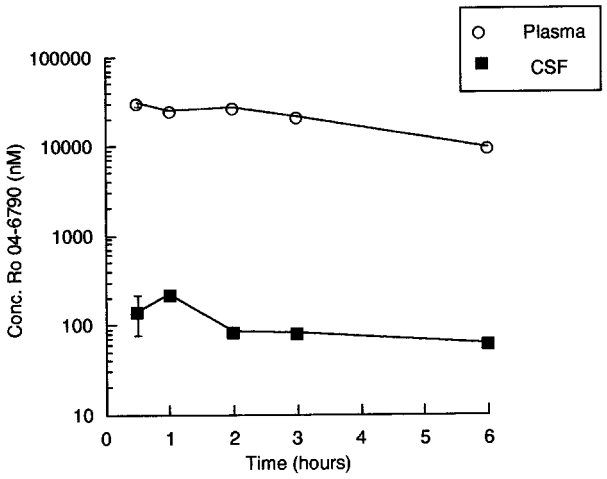

Figure 6 Mean concentrations of Ro 04-6790 in rat plasma and CSF at various time points following administration of Ro 04-6790 (30 mg kg^{-1}, i.p.). Separate rats were used for measurements at each time point. Data points at 0.5 h are the mean$\pm$s.e.mean of determinations from 3 rats, at the 1 h time point, 2 rats were used and at all other time points 1 rat was used.

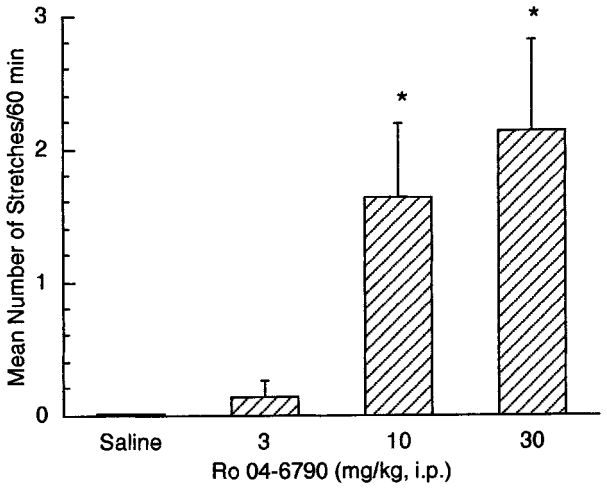

Figure 7 Effect of treatment with either saline, Ro 04-6790 (3 mg kg^{-1}, i.p.), Ro 04-6790 (10 mg kg^{-1}, i.p.) or Ro 04-6790 (30 mg kg^{-1}, i.p.) on the number of stretches (mean$\pm$s.e.mean) counted for a period of 1 h immediately after treatment with the 5-HT$_6$ antagonist. Eight animals were used per treatment group and animals were habituated to the observation cages for a 1 h period on each of the 4 days preceding treatment. In order to minimize the stress effects of injections on the test day, the animals were injected with saline (i.p.) on each of the habituation days. $^*P<0.05$.

Discussion

The present study describes two compounds, Ro 04-6790 and Ro 63-0563, which are potent and highly selective antagonists for the cloned 5-HT$_6$ receptor (Monsma *et al*., 1993; Ruat *et al*., 1993; Kohen *et al*., 1994; Boess *et al*., 1997). Both compounds had similar binding affinities at the rat and human 5-HT$_6$ receptor showing that there are no species differences between the human and the rat receptors for these compounds. Furthermore, both compounds were highly selective for the 5-HT$_6$ receptor, with Ro 04-6790 having over 100 fold selectivity with respect to 23 other binding sites including 8 5-HT receptors. The same was true for Ro 63-0563 which was also tested in a further 45 binding assays by CEREP. In total, Ro 63-0563 was tested in 69 binding assays and was found to have over 100 fold selectivity for the 5-HT$_6$ receptor. When studied in functional assays, neither compound had any significant effect on basal cyclicAMP accumulation, suggesting that they are not either agonists or inverse agonists. However, both compounds behaved as competitive antagonists at the human 5-HT$_6$ receptor with pA$_2$ values of 6.75$\pm$0.07 and 7.10$\pm$0.09, respectively. These values are in agreement with the binding affinities of the compounds at the human 5-HT$_6$ receptor (7.26$\pm$0.06 and 7.91$\pm$0.02, respectively).

The 5-HT$_6$ receptor was identified by molecular biological methods and its pharmacology made the study of naturally occurring 5-HT$_6$ receptors very difficult. All compounds previously identified as having high affinity for the 5-HT$_6$ receptor were non-selective ligands. Potent agonists at the receptor are LSD, ω-N-methyl-5-HT, bufotenine, 5-methoxytryptamine, 5-HT, 2-methyl-5-HT, 5-benzyloxytryptamine and tryptamine all of which are non-selective, having affinities at other 5-HT receptors (Boess *et al*., 1997). LSD, lisuride, 2-methyl-5-HT, tryptamine and 5-benzloxytryptamine behaved as partial agonists compared to 5-HT. Potent antagonists at the receptor are also non-selective and include methiothepin, clozapine, mianserin and ritanserin (Boess *et al*., 1997). Therefore, the identification of physiological responses mediated by the 5-HT$_6$ receptor has not been possible and responses with properties similar to that of the cloned receptor can only be classified as 5-HT$_6$-like (Sleight *et al*., 1997). Indeed, with respect to receptor nomenclature the fact that no selective 5-HT$_6$ ligands existed excluded the 5-HT$_6$ receptor from being officially recognised as a functional, naturally occurring 5-HT receptor (Hoyer *et al*., 1994; Hoyer & Martin, 1997). We propose that responses can now be classified as mediated by the 5-HT$_6$ receptor with the use of Ro 04-6790 and Ro 63-0563. For example, *in vitro* studies have suggested that naturally occurring 5-HT$_6$-like receptors are present in mouse neuroblastoma-derived cell lines N18TG2 and NCB-20 (MacDermot *et al*., 1979; Berry-Kravis & Dawson, 1983; Conner & Mansour, 1990; Unsworth & Molinoff, 1994). However, this classification was only based on a comparison of the rank order of potency of compounds in the functional models with that at the recombinant receptor. In addition, 5-HT$_6$-like receptors have also been demonstrated in pig caudate membranes and mouse striatal neurones in culture where they stimulate cyclicAMP accumulation (Schoeffter & Waeber, 1994; Sebben *et al*., 1994).

The lack of selective tools has also meant that the pharmacological evaluation of the function of 5-HT$_6$ receptors *in vivo* has not been possible. The only study investigating the role of 5-HT$_6$ receptors *in vivo* used i.c.v. injections of antisense oligonucleotides to reduce the expression of the 5-HT$_6$ receptor protein. This treatment gave rise to a behavioural syndrome of yawning, stretching and chewing and caused a 30% reduction in the number of [^{3}H]-LSD binding sites (measured in the presence of 300 nM spiperone). This behavioural syndrome was dose-dependently antagonized by atropine suggesting a modulatory role for 5-HT$_6$ receptors on cholinergic neurones (Bourson *et al*., 1995; Sleight *et al*., 1996). In order to determine whether a 5-HT$_6$ antagonist will produce the same behavioural syndrome, the ability of both Ro 04-6790 and Ro 63-0563 to cross the blood-brain barrier was determined by measuring the CSF and plasma concentrations of the two compounds. Ro 04-6790 but not Ro 63-0563 could be detected in the CSF at concentrations that would occupy more than 70% of the 5-HT$_6$ receptors after administration of 30 mg kg^{-1}, i.p. Interestingly Ro 04-6790 produced a similar behavioural syndrome to that produced by the 5-HT$_6$ antisense

consisting of yawning, stretching and chewing (Figure 7). The stretching behaviour was statistically significant while the yawning just failed to reach statistical significance. Since the behavioural syndrome is produced by both antisense oligonucleotide treatment and selective 5-HT$_6$ antagonists, we conclude that it is indeed mediated by 5-HT$_6$ receptor blockade. It should also be noted that the maximal effect of Ro 04-6790 at 30 mg kg^{-1}, i.p. would also agree with its concentration in the CSF being sufficient to occupy 5-HT$_6$ receptors. These data suggest that experiments with antisense oligonucleotides can be of use in the study of newly cloned receptors where no selective compounds are available, provided that correct controls and techniques are used.

Currently, there is no good binding assay to label 5-HT$_6$ receptors in animal tissue although it has been suggested that [^{3}H]-clozapine labels two populations of receptors in the rat brain, one of which may be the 5-HT$_6$ receptor binding site (Glatt *et al*., 1995). However, 5-HT had no measurable affinity for these binding sites and [^{3}H]-clozapine was not observed in the corpus striatum. The presence of 5-HT$_6$ receptor binding sites in this brain region would have been predicted from *in situ* hybridization studies (Ward *et al*., 1995) and from the mapping of 5-HT$_6$-like immunoreactivity with polyclonal antibodies (Gerard *et al*., 1996). Therefore, the lack of affinity for 5-HT and the localization of [^{3}H]-clozapine binding raises questions as to the validity of this radioligand binding assay. Consequently, it would be interesting to radiolabel a compound such as Ro 63-0563 in order to determine whether there are indeed specific 5-HT$_6$ receptor binding sites in the brain. This work has been started in our group and is now ongoing.

In summary, the present study describes and characterizes two potent and selective 5-HT$_6$ receptor antagonists that can be used to study and characterize the functional role of naturally occurring 5-HT$_6$ receptors. Perhaps, more importantly such compounds may now allow the identification of possible therapeutic uses of 5-HT$_6$ receptor ligands.

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