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RS 39604: a potent, selective and orally active 5-HT_4 receptor antagonist

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1 Selective antagonism of 5-HT_4 receptors may provide therapeutic benefit in certain disorders of the myocardium, alimentary and lower urinary tract. We now report on RS 39604, a novel and selective 5-HT_4 receptor antagonist and compare its pharmacological properties with those of SB 204070.

2 In guinea-pig striatal membranes, both RS 39604 and SB 204070 inhibited specific binding of [^{3}H]-GR 113808 in a concentration-dependent manner yielding pK_i estimates of 9.1 and 10.9, respectively. RS 39604 displayed a low affinity ($pK_i < 6.5$) for 5-HT_{1A}, 5-HT_{2C}, 5-HT_3, α_{1c}, D_1, D_2, M_1, M_2, AT_1, B_1 and opioid μ receptors and moderate affinity for σ_1, ($pK_i = 6.8$) and σ_2 ($pK_i = 7.8$) sites.

3 In the rat isolated oesophagus, precontracted with carbachol, RS 39604 (30–300 nM) behaved as a competitive antagonist towards 5-HT-induced relaxation ($pA_2 = 9.3$; Schild slope = 1.0). We and others have shown previously that SB 204070 behaves as an unsurmountable antagonist in this preparation ($pA_2 \sim 10.5$). In the guinea-pig isolated ileal mucosa, RS 39604 (30 nM) antagonized 5-MeOT-induced increase in short-circuit current ($pA_2 = 9.1$).

4 In anaesthetized vagotomized micropigs, RS 39604, administered by the i.v. or intraduodenal (i.duod.) route, produced dose-dependent inhibition of 5-HT-induced tachycardia ($ID_{50} = 4.7$ μg kg^{-1}, i.v. and 254.5 μg kg^{-1}, i.duod). At maximal doses of 30 μg kg^{-1}, i.v. and 6 mg kg^{-1}, i.duod., the inhibitory effects of RS 39604 lasted for more than 6 h. In this preparation, SB 204070 was as potent as RS 39604 by the i.v. route but was inactive by the intraduodenal route at doses up to 3 mg kg^{-1}.

5 In conscious mice, RS 39604, administered by the i.p. or p.o. route, produced dose-dependent inhibition of 5-hydroxytryptophan (5-HTP)-induced diarrhoea ($ID_{50} = 81.3$ μg kg^{-1}, i.p. and 1.1 mg kg^{-1}, p.o.). In this assay, SB 204070 was inactive by the oral route at doses up to 30 mg kg^{-1}.

6 In anaesthetized guinea-pigs, RS 39604 antagonized the contractile effect of 5-HT in the proximal colon by producing parallel, dextral displacement of the dose-response curve to 5-HT. The mean dose-ratios to 5-HT at 0.1 mg kg^{-1}, i.v., 1 mg kg^{-1}, i.v. and 10 mg kg^{-1}, i.duod. were 4.6, 30.7 and 10.8, respectively. SB 204070 behaved as an unsurmountable antagonist in this assay.

7 In a model of visceral pain in conscious rats, RS 39604 (0.01–1 mg kg^{-1}, i.v.) did not affect colorectal distension-induced increases in arterial pressure whereas morphine (1 mg kg^{-1}, i.v.) produced significant inhibition of the response, implying that 5-HT_4 receptors are not involved in nociception in this model.

8 The data suggest that RS 39604 is a high affinity and selective 5-HT_4 receptor antagonist that is orally active and long-lasting *in vivo*. It is concluded that RS 39604 may be the preferable probe to use for investigating the physiological and pathophysiological role of 5-HT_4 receptors *in vivo*.

Keywords: 5-HT_4 antagonist; RS 39604; SB 204070; rat oesophagus; micropig tachycardia; short-circuit current; mice diarrhoea; guinea-pig colon; visceral pain; colorectal distension

Introduction

Significant advances have been made in our understanding of the physiology and pharmacology of the 5-HT_4 receptor (see Ford & Clarke, 1993 for review). Of particular clinical significance are the findings which show that this receptor mediates several biological responses in human tissues. In human gut for example, 5-HT_4 receptors have been shown to mediate 5-HT-induced inhibition of spontaneous colonic circular muscle contractions (Tam *et al.*, 1994) and stimulation of short circuit current responses in ileal (Burleigh & Borman, 1993) and jejunal (Budhoo & Kellum, 1994) mucosa. In the lower urinary tract of man, 5-HT-induced potentiation of cholinergic transmission in the detrussor smooth muscle is also mediated by 5-HT_4 receptors (Tonini *et al.*, 1994). Furthermore, 5-HT can augment aldosterone secretion from the human adrenal cortex via activation of 5-HT_4 receptors (Lefebvre *et al.*, 1993) while agonism of 5-HT_4 receptors in the human atrium can evoke pronounced arrythmias (Kaumann & Sanders, 1994). Based on these findings and assuming a pathophysiological role of endogenous 5-HT in these tissues, it may be hypothesized that selective antagonism of 5-HT_4 receptors might be of therapeutic value notably in the treatment of irritable bowel syndrome, urinary incontinence or cardiac arrythmias.

Until recently, tropisetron (Dumuis *et al.*, 1989), DAU 6285 (Dumuis *et al.*, 1992) and SDZ 205-557 (Buchheit *et al.*, 1991) were the only compounds available that antagonized the 5-HT_4 receptor. However, these compounds, had the drawbacks of low affinity and poor preference for the 5-HT_4 receptor. Subsequently, RS 23597-190 (Eglen *et al.*, 1993) and GR 113808 (Gale *et al.*, 1994a) were identified as antagonists possessing a high affinity ($pA_2 = 7.8$ and 9.2, respectively) and selectivity (> 100 fold over other 5-HT receptors) for 5-HT_4 receptors. These two compounds, although useful as pharmacological tools, were of limited therapeutic value owing to their extremely short biological half-life (Eglen *et al.*, 1993; Gale *et al.*, 1994a). More recently, SB 204070 (Wardle *et al.*, 1994) and GR 125487 (Gale *et al.*, 1994b) were reported to antagonize the 5-HT_4 receptor with high affinity ($pK_B > 10.0$) and selectivity (> 1000 fold over other 5-HT receptors). These compounds have been used successfully to antagonize 5-HT_4 receptors *in vivo* even when administered as a single bolus intravenous dose (Banner *et al.*, 1993; Bingham *et al.*, 1993; Gale *et al.*, 1994c) although their efficacy by the oral route has not

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been reported. Furthermore, GR 125487 has been found to have a long duration of action (75 and 145 min in piglet and rat, respectively) (Gale *et al.*, 1994c) although similar data for SB 204070 are lacking.

In the present study, we have assessed the pharmacological properties of RS 39604, a novel 5-HT$_4$ receptor antagonist (Figure 1), and compared it to those of SB 204070. RS 39604, in contrast to SB 204070 and GR 125487, lacks a carboxylic ester functional group (Clark *et al.*, 1994) and was designed with the intent of improving the metabolic stability of our earlier chemical leads, such as RS 23597 (Eglen *et al.*, 1993).

A preliminary account of the findings has been presented to the British Pharmacological Society, (Hegde *et al.*, 1995).

Methods

In vitro *studies*

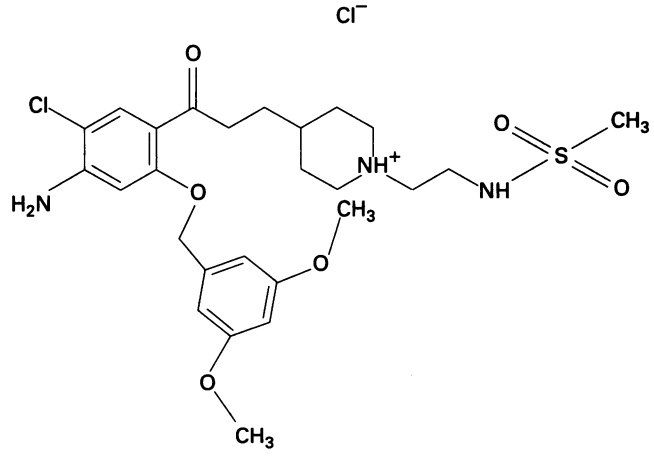
Radioligand binding studies The affinity of compounds at 5-HT$_4$ receptors was assessed in binding studies using [^{3}H]-GR 113808 as radioligand (Grossman *et al.*, 1993). [^{3}H]-GR 113808 binding to 5-HT$_4$ receptors was measured in a synaptosomal membrane preparation of guinea-pig striatum, obtained from animals previously killed by CO_2 asphyxiation. Striata were homogenized with a hand driven glass homogenizer in a Tris (10 mM)-EDTA (5 mM) buffer (pH 7.4 at 4°C), containing 250 mM sucrose. The homogenate was filtered through a nylon mesh (160 μm pore) and then centrifuged at 1000 *g* for 15 min. The resulting pellet was suspended in a HEPES (50 mM)-EDTA (0.5 mM) buffer (pH 7.4 at 4°C) containing choline (130 mM), glucose (25 mM) and KCl (5.4 mM). The final pellet was resuspended in a Tris (25 mM) buffer (pH 7.4 at 4°C).

Binding assays were conducted in a Tris buffer (25 mM) with approximately 0.1 mg striatal protein in an assay volume of 0.5 ml at room temperature. Non-specific binding was determined with 1 μM unlabelled GR 113808. Preliminary studies have demonstrated that a 60 min incubation was sufficient for membrane binding to reach a steady state. Competition binding studies were conducted with 0.1 nM [^{3}H]-GR 113808 and varying concentrations of competing ligand. Reactions were terminated by vacuum filtration, using a Brandel cell harvester, through GF/B filters pretreated for 30 min with 0.1% polyethylenimine. Filters were then dried and the bound radioactivity determined.

The affinity of RS 39604 for a range of neurotransmitter receptors were also determined (Table 1). Details of the individual assays can be found in Wong *et al.* (1993) and references cited therein.

Rat isolated oesophageal muscularis mucosae The method used was similar to that described earlier (Baxter *et al.*, 1991). The thoracic oesophagus was isolated from male Sprague-Dawley rats (Charles River, MA, U.S.A., 200–250 g) and placed in Tyrode solution (composition, mM: NaCl 139.0, KCl 2.7, $MgCl_2$. $6H_2O$ 1.1, NaH_2PO_4 0.4, glucose 5.6, $NaHCO_3$ 11.8 and $CaCl_2$. $6H_2O$ 1.8). The outer striated muscle coat was cut longitudinally and gently peeled away to expose the inner muscularis mucosae. Tissues were then mounted vertically in 10 ml organ baths containing Tyrode solution, maintained at 37°C and gassed with 95% O_2/5% CO_2. Methysergide (1 μM) was routinely included in the Tyrode solution in order to block 5-HT$_1$ and 5-HT$_2$ receptors. In addition, cocaine (30 μM) and corticosterone (30 μM) were also included in the Tyrode solution in order to inhibit amine uptake. An initial resting tension of 1 g was applied to the preparation and readjusted to 0.5 g during the initial equilibration period of 60 min. After this period, pargyline (100 μM a monoamine oxidase inhibitor) was added to the Tyrode solution for 30 min, followed by a washout period. The tissues were then allowed to equilibrate for 30 min. Carbachol (3 μM) was added to contract the tissues. Upon establishment of a stable contraction, a cumulative concentration-effect curve to 5-HT was constructed. The tissues were then washed and equilibrated for 60 min in the presence of the appropriate concentration of the antagonist. A second concentration-effect curve to 5-HT was then constructed in the presence of the antagonist.

Short circuit current studies in guinea-pig ileal mucosa Male Hartley guinea-pigs (Charles River, MA, U.S.A., 300–350 g) were killed by CO_2 asphyxiation and a segment of distal ileum, beginning approximately 2.0 cm proximal to the ileocecal valve, was isolated. Ileal mucosal sheets were prepared and mounted in Ussing chambers (surface area of 0.6 cm^2) for the measurement of short circuit current (I_{sc}) as described previously (Cooke & Carey, 1985; Scott *et al.*, 1992). The tissues were bathed, on both sides, in 15.5 ml of Krebs-Henseleit solution (composition, in mM: NaCl 118.2, KCl 4.6, $MgSO_4$. $7H_2O$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2, $CaCl_2.6H_2O$ 1.8 at 37°C and gassed with 95% O_2/5% CO_2). Glucose (10 mM) was added to the serosal solution and mannitol (10 mM) to the mucosal solution. The tissue generated potential was clamped at 0 mV using a voltage clamp amplifier (DVC 1000, World



RS-39604

Figure 1 Chemical structure of RS 39604 (1-(4-amino-5-chloro-2-(3,5-dimethoxy)benzyloxyphenyl)-3-[1-((2-methylsulfonylamino)ethyl)-piperidin-4-yl]-1-propanone).

Table 1 Binding profile of RS 39604 at various receptors in radioligand binding assays

Receptor	pK_i
Adrenoceptor α_{1C}	6.4 ± 0.04
Dopamine D$_1$	< 6
Dopamine D$_2$	< 6
Muscarinic M$_1$	< 6
Muscarinic M$_2$	< 6
5-HT$_{1A}$	< 6
5-HT$_{2C}$	6.1 ± 0.05
5-HT$_3$	6.0 ± 0.2
5-HT$_4$	9.1 ± 0.1
Angiotensin AT$_1$	< 5
Bradykinin B$_1$	< 5
Opioid μ	< 6
NMDA channel	< 6
σ_1	6.8 ± 0.1
σ_2	7.8 ± 0.1

Values are means ± s.e.mean, $n = 3-4$. In all experiments the Hill coefficients were not significantly different from unity.

Precision Instruments, FL, U.S.A.). The I_{sc} required to effect a 0 mV clamp was recorded on a MacLab data acquisition system. All I_{sc} values are expressed as $\mu A\ cm^{-2}$.

Tissues were equilibrated for 60 min in the presence of vehicle or the appropriate concentration of the antagonist prior to the construction of the cumulative concentration-effect curve to 5-methoxytryptamine (5-MeOT). A single concentration-effect curve was obtained in each tissue because of agonist-induced desensitization. Adjacent tissues were used for the vehicle and drug-treatment groups. Drug additions were made to both sides of the tissue cumulatively at half log increments. Carbachol (10 μM) was added to the tissues at the end of experiments to establish tissue viability.

In vivo *studies*

5-HT-induced-tachycardia in anaesthetized micropigs The method used was modified from that described by Villalon *et al.* (1990). Yucatan micropigs of either sex (17–22 kg, S & S Farms, CA, U.S.A.) were pretreated with ketamine (approx 30 mg kg^{-1}, i.m.), anaesthetized with pentobarbitone sodium (20 mg kg^{-1}, i.v.), intubated and mechanically ventilated with room air by an animal respirator (Harvard, model 613). A femoral artery was cannulated for measurement of arterial blood pressure via a pressure transducer (Gould Statham P23ID). Dual cannulae were inserted in the ipsilateral femoral vein, one cannula for continuous infusions of supplemental anaesthetic and the second cannula for drug administration. In some experiments, a cannula was introduced into the duodenum, following a laparotomy, to allow intraduodenal administration of drugs. A limb lead II ECG electrode was monitored by subcutaneously placed electrodes and heart rate was determined by a cardiotachometer triggered by the R wave of the ECG. Following a midline ventral incision, vagus nerves were cut bilaterally. Normal body temperature was maintained with a heated water blanket. Blood gas parameters were stabilized with in a normal physiological range (pH 7.4–7.5; PCO_2 30–40, PO_2 80–100 mmHg) by adjustments of ventilatory rate, tidal volume and positive end expiratory pressure prior to the commencement of the experiment.

A dose-response curve to 5-HT (1–100 μg kg^{-1}, i.v.) was constructed for each animal to establish the ED_{50} (dose required to produce 50% of the maximal tachycardic response) for 5-HT. After obtaining three reproducible control responses to 5-HT (ED_{50} dose), each animal received (i.v. or i.d.) either vehicle or increasing cumulative doses of the antagonist with i.v. and i.d. dosing intervals of 30 and 60 min, respectively. After each dose of the antagonist or vehicle, the animal was challenged with 5-HT at 5 and 15 min for the i.v. studies and 15, 30 and 45 min for the i.duod. studies.

In a separate series of experiments, we sought to determine the duration of action of the antagonists. After obtaining three control tachycardic responses to 5-HT (ED_{50} dose), each animal was treated (i.v. or i.duod.) with either vehicle or the appropriate dose of the antagonist. For the i.v. duration study, each animal was challenged with 5-HT at 3, 10, 20 and 30 min post-dose and every 15 min thereafter for the next 330 min. For the i.d. duration study, each animal was challenged with 5-HT at 15, 30, 45 and 60 min post-dose and every 30 min thereafter for the next 420 min.

5-Hydroxytryptophan-induced diarrhoea in mice The method used was similar to that described previously (Hegde *et al.*, 1994b). Adult male Swiss-Webster mice (Harlan, San Diego, CA, U.S.A., 18–25 g) were used. The animals had free access to food and water prior to the experiments. On the day of the experiment, mice were pre-screened to exclude animals with pre-existing diarrhoea. The selected mice were randomly divided into appropriate groups. Each animal was treated intraperitoneally (i.p.) with either vehicle or the appropriate dose of antagonist. Five min later, each animal was treated with either saline or 5-hydroxytryptophan (5-HTP, 10 mg kg^{-1}, i.p.) and immediately placed in a 1 litre cup. The animals were continuously monitored for 30 min. The severity of diarrhoea was assessed using an arbitary scoring scale from 0 to 3; 0 = normal stools, 2 = swollen and/or unformed stools, 3 = severe watery diarrhoea. In a separate series of experiments, the effect of orally administered antagonist was determined. In these experiments, the animals were pre-treated with either vehicle or the appropriate dose of antagonist 10 min before 5-HTP.

In a separate series of experiments, the effect of antagonist on 16, 16, dimethyl PGE_2-induced diarrhoea was determined. Each mouse was treated with vehicle or the appropriate dose of the antagonist. Five min later, each mouse was treated with either saline or 16, 16, dimethyl PGE_2 (30 μg kg^{-1}, i.p.) and immediately placed in a 1 litre cup. The severity of diarrhoea was then scored over a 30 min observation period.

5-HT-induced contraction of the ascending colon in anaesthetized guinea-pigs The method used was similar to that described previously (Hegde *et al.*, 1994a). Male Hartley guinea-pigs (Charles River, Willmington, MA, U.S.A., 350–450 g) were used for the study. The animals were fasted, with water allowed *ad libitum* 18 h before the experiment. The animals were anaesthetized with inactin (80 mg kg^{-1}, i.p.) and the trachea was cannulated to allow artificial respiration with a ventilator (Harvard Apparatus, Model 683). The abdominal viscera were exposed and a miniature strain guage (Biomedical Dynamics, CA, U.S.A.) was sutured on the ascending colon. The strain guage, oriented to measure predominantly circular muscle tension, was connected to a signal conditioning amplifier (Measurement group, NC, U.S.A.) and a recorder (Beckman, Model 611) for measurement of tension. The left jugular vein and, in some cases, the duodenum were cannulated to allow intravenous and intraduodenal administration of drugs, respectively. Following a 1 h stabilization period, baseline tension was recorded for about 45–60 min. Each animal was treated with methysergide (3 mg kg^{-1}, i.v.) and granisetron (1 mg kg^{-1}, i.v.) in order to block 5-HT_1, 5-HT_2 and 5-HT_3 receptors. In addition, the animals were treated (i.v. or i.duod.) with either vehicle or the appropriate dose of the 5-HT_4 receptor antagonist. Sixty min later, a non-cumulative dose-effect curve to 5-HT (0.3–1000 μg kg^{-1}, i.v.) was constructed. At the end of the dose-effect curve, carbachol (100 μg kg^{-1}, i.v.) was administered to each animal.

Colorectal distension model of visceral pain in conscious rats The method used was modified from that described previously (Ness & Gebhart, 1988). Male Sprague-Dawley rats (Harlan, San Diego, CA, U.S.A., 250–300 g) were used for the study. The animals were fasted 18 h before the experiment but allowed water *ad libitum*. Each animal was anaesthetized with ether and the femoral artery and vein were cannulated for measurement of arterial pressure and drug administration, respectively. A latex balloon catheter (Viggo-Spectramed, Model SO 5325H) was introduced into the descending colon via the anus. The animals were transferred to restrainers and allowed to recover from anaesthesia (60–90 min). The arterial catheter was connected to a pressure transducer (Gould P23XL) and arterial pressure was recorded on a Beckman (Model 611) recorder. Colorectal distension was accomplished by air-inflation of the latex balloon for a period of 20 s which induced a reproducible increase in arterial pressure. After obtaining two control colorectal distension-induced pressor responses (10 min apart), each animal was treated intravenously with either vehicle or ascending cumulative doses of the antagonist. A colorectal distension-induced response was obtained 10 min after each dose of antagonist or vehicle. After the last dose, morphine (1 mg kg^{-1}, i.v.) was administered to each animal. Ten min later, another colorectal distension-induced pressor response was obtained.

Data analysis and statistical methods

Radioligand binding studies In radioligand binding studies, data from competition binding studies were analysed by fitting

to a four parameter logistic function using an iterative curve fitting program. The apparent affinities (–log K_i) of competing ligands were calculated from IC_{50} values by the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Functional isolated tissue studies In the functional studies, agonist potencies were determined by nonlinear regression using iterative curve fitting procedures (Leung *et al.*, 1992) and the relationship described by Parker & Waud (1971). The apparent affinity (pA_2) of the antagonist was determined by the method of Arunlakshana & Schild (1959), in which at least three concentrations of antagonist were used and the slope of the Schild plot determined by regression analysis. In studies where only a single concentration of the antagonist was used, the apparent affinity was calculated by the method described by Furchgott (1972).

Micropig tachycardia studies In these experiments, peak percentage inhibition of the control tachycardic response to 5-HT was determined for each antagonist. ID_{50} estimates (dose required to produce 50% of the maximal inhibition) were obtained by non-linear iterative curve fitting procedures (Leung *et al.*, 1992). In the duration studies, a repeated measures two-way analysis of variance (ANOVA) was used to determine the effects of treatment, time and their interaction. Wherever appropriate, a 2-parameter exponential model was used to determine $t_{1/2}$ (time required for the inhibitory effect to decline by 50%).

5-HTP-induced diarrhoea in mice The scorer was blind to treatments. A Mantel-Haenszel Chi-square test was used to analyse the effects of different treatments on diarrhoea score. ID_{50} estimates (dose required to produce 50% of the maximal inhibition of 5-HTP-induced diarrhoea) were calculated by non-linear regression analysis (Leung *et al.*, 1992).

5-HT-induced colonic contraction in guinea-pigs The contractile response to 5-HT in each animal was expressed as a percentage of the response to carbachol. ED_{50} (dose required to produce 50% of the maximum response) and E_{max} (maximum response) of 5-HT were estimated by nonlinear regression analysis (Leung *et al.*, 1992). Dose-ratios were calculated as the ratio of ED_{50} (antagonist)/ED_{50} (vehicle). In cases where the antagonist produced marked depression of the maximum response to 5-HT, an alternate potency estimate (ED_x) was calculated for the vehicle curve. ED_x was defined as the concentration of 5-HT required to attain a response equivalent to 50% of the maximum response of the antagonist curve. Dose-ratio was then calculated as ED_{50} (antagonist)/ED_X (vehicle).

Colorectal distension model of visceral pain in rats The change in mean arterial pressure (from pre-distension baseline) following each trial of colorectal distension were obtained. The difference between the control response and post-drug (or vehicle or morphine) response was calculated. A repeated measures two-way ANOVA was used to test the overall effects of treatment, dose and their interaction. One way ANOVA was also performed to test the effects of each dose of antagonist or morphine.

All values are either mean ± s.e.mean or mean, with 95% confidence intervals in parentheses. $P < 0.05$ was considered to be statistically signficant.

Compounds used

The following compounds were obtained from commercial sources: 5-HT, 5-MeOT, cocaine, corticosterone, pargyline, carbachol, 16, 16, dimethyl PGE_2 (Sigma Chemical Co, St. Louis, MO, U.S.A.), 5-HTP (Research Biochemicals Inc, MA, U.S.A.). Methysergide was donated by Sandoz, Basle, Switzerland. RS39604 (1-(4-amino-5-chloro-2-(3,5-dimethoxy)benzyloxyphenyl)-3-[1-((2-methylsulphonylamino)ethyl)piperidin-4-yl]-1-propanone), SB 204070 ((1-butyl-4-piperidiny)methyl-8-amino-7-chloro-1,4-benzodioxan-5-carboxylate hydrochloride) and granisetron were synthesized in the Institute of Organic Chemistry at Syntex Discovery Research.

5-HT, 5-MeOT, 5-HTP, cocaine, carbachol, pargyline and 16, 16, dimethyl PGE_2 were dissolved in either distilled water or saline. Corticosterone was dissolved in dimethyl sulphoxide (DMSO). Methysergide was dissolved in saline or ethanol. RS 39604 and SB 204070 were dissolved in either 50% ethanol (for the *in vitro* experiments) or a mixture of DMSO, ethanol and water (for the pig tachycardia experiments) or in a solution of 4.5% 2-hydroxypropyl β-cyclodextrin for the remaining experiments.

Results

In vitro *studies*

Radioligand binding studies In guinea-pig striata, RS 39604 produced concentration-dependent displacement of [^{3}H]-GR 113808 ($pK_i \pm$ s.e.mean = 9.1 ± 0.1; Hill slope ± s.e.mean = 1.1 ± 0.1). RS 39604 displayed a low affinity ($pK_i < 6.5$) for several other receptors except the σ_1 ($pK_i = 6.8$) and σ_2 site ($pK_i = 7.8$) (Table 1).

SB 204070 also produced concentration-dependent displacement of [^{3}H]-GR 113808 ($pK_i \pm$ s.e.mean = 10.9 ± 0.1; Hill slope ± s.e.mean = 1.1 ± 0.1).

Rat isolated oesophageal muscularis mucosae 5-HT produced concentration-dependent relaxation of the pre-contracted rat oesophagus with a pEC_{50} (95% confidence interval) of 8.3 (8.1–8.6) (Figure 2). RS 39604 (10–300 nM) had no effect, *per se*, on basal or carbachol-induced tone (data not shown). RS 39604 (30–300 nM) produced concentration-dependent dextral displacement of the concentration-effect curve to 5-HT without significantly altering the maximum response to 5-HT (Figure 2). Schild analysis of this data yielded a pA_2 estimate (95% confidence interval) and slope (± s.e.mean) of 9.3 (9.2–9.4) and 1.0 ± 0.2, respectively.

We and others have shown previously that, in the rat oesophagus, SB 204070 also produces concentration-dependent rightward displacement of the concentration-effect curve to 5-HT which is accompanied by a significant depression of the maximum response ($pA_2 \sim 10.5$) (Baxter *et al.*, 1994; Zeitung *et al.*, 1994).

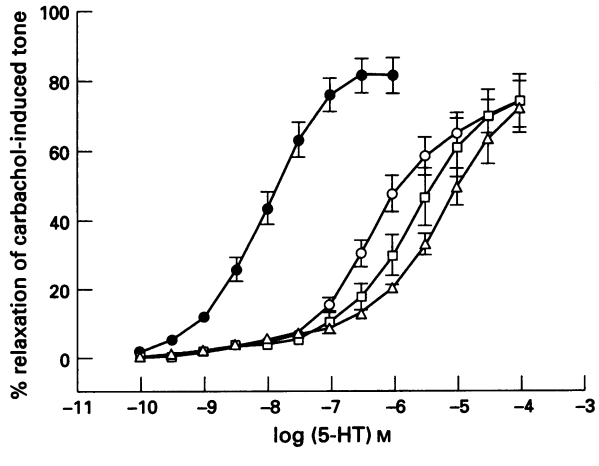


Figure 2 Effect of RS 39604 on the relaxant response to 5-HT in the rat isolated oesophagus. The concentration-effect curve to 5-HT is shown in the absence (●) and presence of RS 39604, 30 nM (○), 100 nM (□) and 300 nM (△). Results are expressed as mean ± s.e.mean, $n = 4$.

Short-circuit current studies in guinea-pig ileal mucosa We have reported, previously, that 5-HT induces a tetrodotoxin-sensitive increase in short-circuit current in this preparation (Eglen *et al.*, 1993). Furthermore, the concentration-effect curve to 5-HT is biphasic with the high potency phase being mimicked by 5-MeOT and abolished by 5-HT$_4$ antagonists such as RS 23597-190. In the present study, 5-MeOT induced a concentration-dependent rise in short-circuit current with a pEC_{50} (95% confidence interval) of 7.5 (6.9–8.0). RS 39604 (30 nM), which did not affect short-circuit current *per se*, produced a dextral shift of the concentration-effect curve to 5-MeOT without significantly altering the maximum response (Figure 3). The pA_2 estimate (95% confidence interval) of RS 39604 was calculated to be 9.1 (7.8–10.3).

In vivo *studies*

Micropig tachycardia studies Both RS 39604 and SB 204070, when administered intravenously, produced dose-dependent inhibition of 5-HT-induced tachycardia in anaesthetized micropigs (Figure 4a and b) with ID_{50} (95% confidence interval) estimates of 4.7 (3.6–6.0) and 8.0 (5.4–11.9) μg kg^{-1}, i.v., respectively. RS 39604, when administered intraduodenally, produced dose-dependent inhibition of 5-HT-induced tachycardia (Figure 4a) with an ID_{50} estimate of 246 (191–324) μg kg^{-1}, i.duod. SB 204070 had no significant inhibitory effects, when administered intraduodenally, at doses up to 3 mg kg^{-1}, i.duod. (Figure 4b). Both SB 204070 and RS 39604, when administered as escalating cumulative i.v. or i.duod. doses, did not affect baseline heart rate or arterial pressure (data not shown).

At maximal inhibitory doses of 30 μg kg^{-1}, i.v. and 6 mg kg^{-1}, i.duod., which did not affect baseline heart rate or arterial pressure *per se*, the inhibitory effects of RS 39604 toward 5-HT were statistically significant ($P < 0.05$) for more than 6 and 8 h, respectively (Figure 5). There appeared to be a temporal increase in the sensitivity of the preparation to 5-HT in animals pre-treated with vehicle. At a dose of 30 μg kg^{-1}, i.v., the $t_{1/2}$ (95% confidence interval) of RS 39604 was calculated to be 315 (278–352) min. SB 204070, when administered as a single intravenous bolus dose of 30 μg kg^{-1}, itself induced a positive chronotropic effect (increase in heart rate of approximately 25–30 beats min^{-1}) which lasted for approximately 90 min and could be partially reversed by DAU 6285 (3 mg kg^{-1}, i.v., data not shown). At 90 min post-dose, at which time baseline heart rate was re-attained, the tachycardic response to exogenous 5-HT was inhibited by approximately 35%.

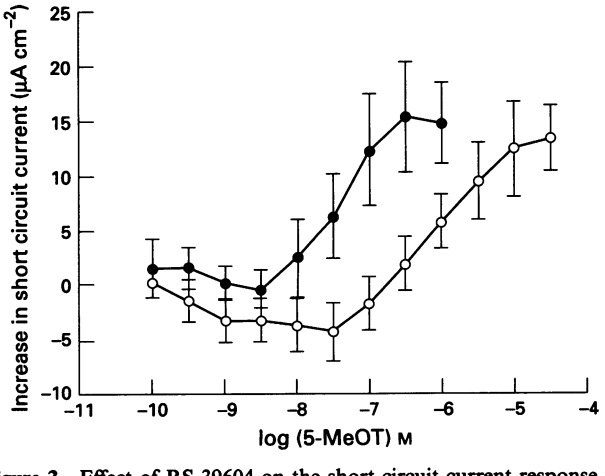


Figure 3 Effect of RS 39604 on the short-circuit current response to 5-methoxytryptamine (5-MeOT) in the guinea-pig isolated ileal mucosa. Figure shows the concentration-effect curve to 5-HT in the absence (●) and presence (○) of RS 39604 (30 nM). Results are expressed as mean ± s.e.mean, $n = 4$.

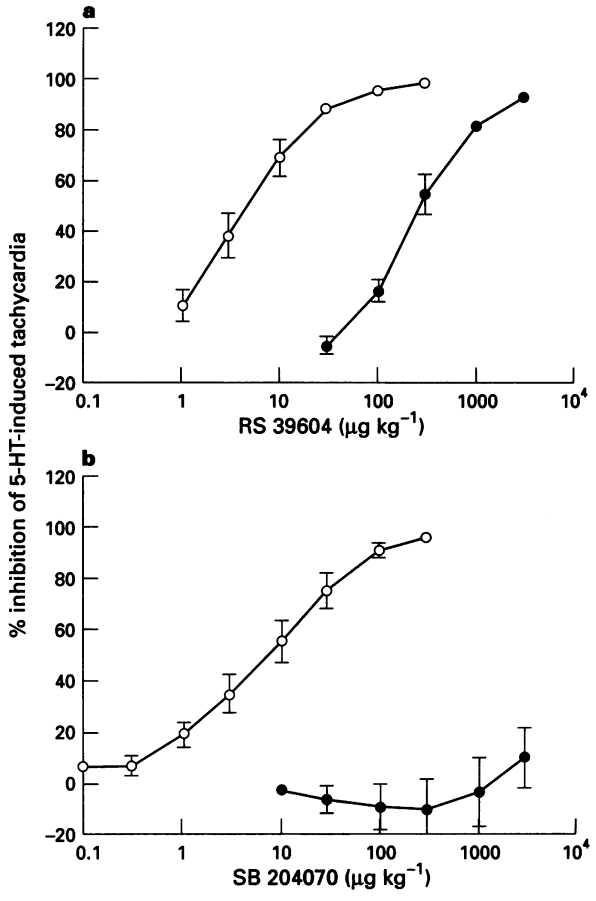


Figure 4 Effect of RS 39604 (a) and SB 204070 (b) on 5-HT-induced tachycardia in the anaesthetized micro-pig. The peak inhibitory effects of intravenously (○) or intraduodenally (●) administered antagonist are shown. The antagonists were administered as increasing cumulative doses. Results are expressed as mean ± s.e.mean, $n = 4$, and show the percentage inhibition of the control (pre-drug) tachycardic response to 5-HT.

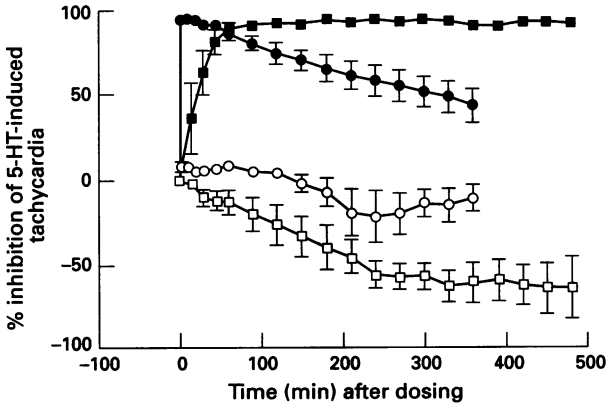


Figure 5 Time course of the effect of vehicle (open symbols) or RS 39604 (closed symbols) on 5-HT-induced tachycardia in the anaesthetized micropig. After obtaining control tachycardic responses to 5-HT, animals were pretreated at 0 min with vehicle (○) i.v., (□) i.duod. or RS 39604, 30 μg kg^{-1}, i.v. (●); 6 mg kg^{-1}, i.d. (■) after which the animals were re-challenged with 5-HT at appropriate intervals. Results are expressed as mean ± s.e.mean, $n = 4$, and show the percentage inhibition of the control (pre-drug or pre-vehicle) tachycardic response to 5-HT. The inhibitory effects of RS 39604 (i.v. and i.d.) were significantly ($P < 0.05$) different from vehicle at all time points.

5-HTP-induced diarrhoea in mice 5-HTP (10 mg kg^{-1}, i.p.) produced a significant increase in diarrhoea score (Figure 6a and 6b). RS 39604, administered i.p. or p.o., produced dose-dependent inhibition of 5-HTP-induced diarrhoea (Figure 6a and 6b). The ID_{50} (±s.e.mean) estimates were 81.3± 1.9 μg kg^{-1}, i.p. and 1.1±4.2 mg kg^{-1}, p.o. The maximal inhibitory effect of RS 39604 by the i.p. and p.o. route were 70% and 58%, respectively. RS 39604, at doses of 0.1 and 1 mg kg^{-1}, i.p., did not affect 16,16, dimethyl PGE_2-induced diarrhoea (data not shown). As reported earlier (Hegde *et al.*, 1994b), SB 204070, when administered intraperitoneally, also produced significant inhibition of 5-HTP-induced diarrhoea. The ID_{50} (±s.e.mean) and maximal inhibitory effect of SB 204070 were 0.3±0.2 μg kg^{-1}, i.p. and 36%, respectively. SB 204070, administered orally, had no inhibitory effects on 5-HTP-induced diarrhoea even at doses up to 30 mg kg^{-1} (data not shown).

5-HT-induced colonic contraction in anaesthetized guinea-pigs RS 39604 (0.1 and 1 mg kg^{-1}, i.v.) produced dose-dependent dextral displacement of the dose-effect curve to 5-HT with no significant alteration of the maximum response (Figure 7a). When administered intraduodenally at 10 mg kg^{-1}, i.p., RS 39604 also produced a significant dextral displacement of the dose-effect curve to 5-HT (data not shown). The mean dose-ratios (95% confidence interval) at 0.1 mg kg^{-1}, i.v., 1 mg kg^{-1}, i.v. and 10 mg kg^{-1}, i.d. were 4.6 (1.9–10.9), 30.7 (8.5–111.3) and 10.8 (2.6–44.4), respectively. SB 204070 (0.03 and 0.1 mg kg^{-1}, i.v.) also produced dose-dependent dextral displacement of the dose-response curve to 5-HT which was accompanied by a significant depression of the maximum response (Figure 7b). The mean dose-ratios (95% confidence interval) at 0.03 and 0.1 mg kg^{-1}, i.v. were 3.9 (1.3–12.6) and 11.2 (4.5–27.8) respectively. Both RS 39604 and SB 204070 had no effect on baseline tension *per se* (data not shown).

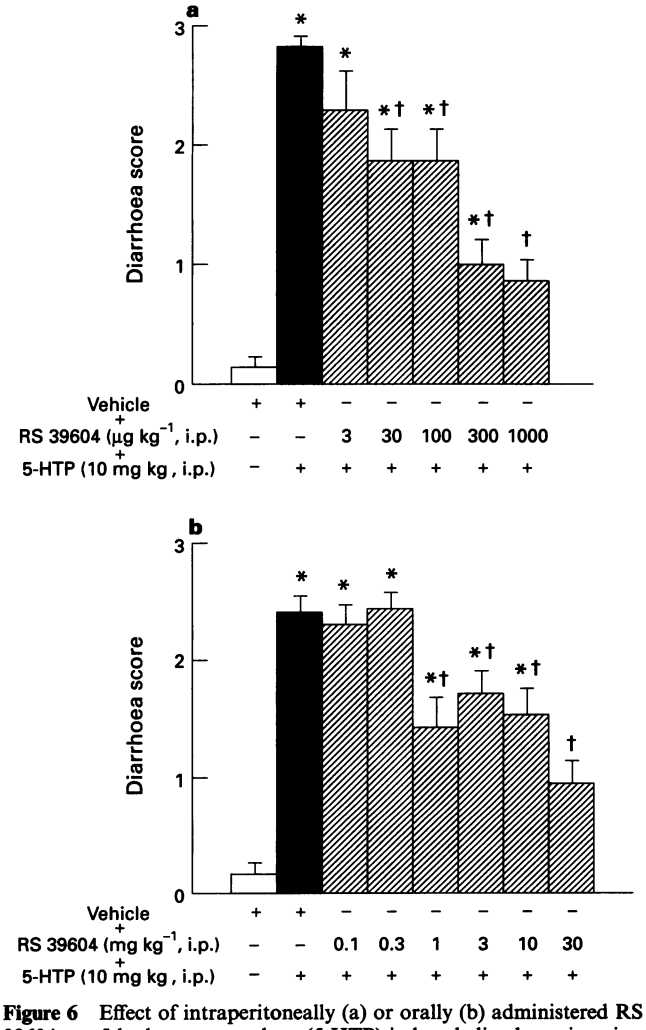


Figure 6 Effect of intraperitoneally (a) or orally (b) administered RS 39604 on 5-hydroxytryptophan (5-HTP)-induced diarrhoea in mice. Figures show the control diarrhoea score (open column), 5-HTP-induced increase in diarrhoea score (solid column) and effect of different doses of RS 39604 on 5-HTP-induced diarrhoea (hatched columns). Results are expressed as mean±s.e.mean, n=30–40. *P<0.05 vs open column; †P<0.05 vs closed column.

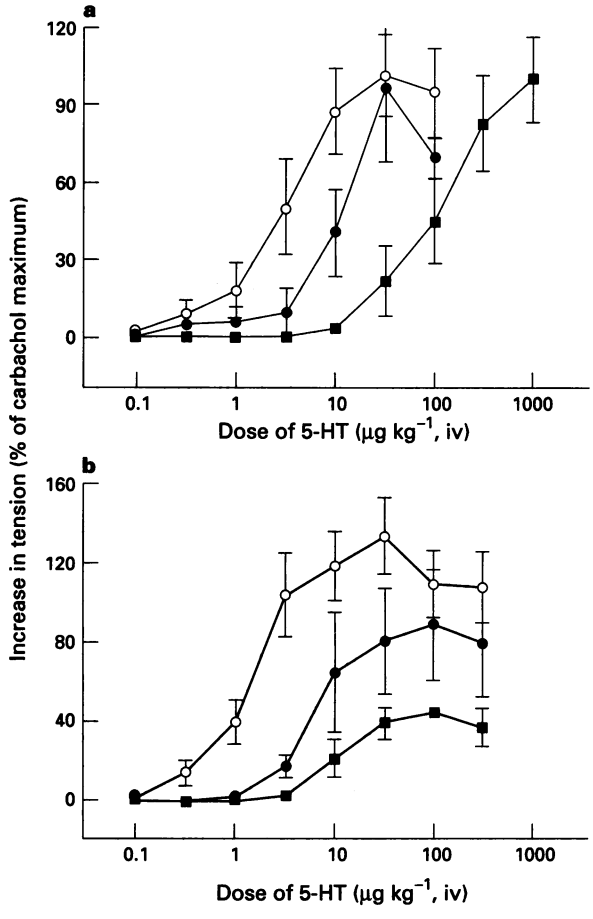


Figure 7 Effect of RS 39604 (a) and SB 204070 (b) on the contractile effects of 5-HT in the proximal colon of the anaesthetized guinea-pig: (a) shows the dose-effect curve to 5-HT in the absence (○) and presence of RS 39604, 0.1 mg kg^{-1}, i.v. (●) and 1 mg kg^{-1}, i.v. (■); (b) shows the dose-effect curve to 5-HT in the absence (○) and presence of SB 204070, 0.03 mg kg^{-1}, i.v. (●) and 0.1 mg kg^{-1}, i.v. (■). Results are expressed as mean±s.e.mean, n=5–6.

Table 2 Effect of RS 39604 and morphine on colorectal distension-induced increase in arterial pressure in rats

Treatment	*Dose* (mg kg^{-1}, i.v.)	*Change in arterial pressure* (mmHg)
Control	NA	30.6±5.3
RS 39604	0.01	31.6±5.2
	0.03	26.2±5.9
	0.1	31.0±6.4
	0.3	31.8±4.4
	1.0	29.2±5.4
Morphine	1.0	17.8±2.2*

NA=not applicable; Results are expressed as mean± s.e.mean, n=5.
*P<0.05 vs control.

Colorectal distension model of visceral pain in conscious rats In vehicle-treated rats, colorectal distension evoked reproducible increases in arterial pressure (data not shown). RS 39604 (0.01–1 mg kg^{-1}, i.v.) had no effect on colorectal distension-induced pressor responses (Table 2). In contrast, morphine (1 mg kg^{-1}, i.v.) significantly inhibited the pressor response to colorectal distension (Table 2).

Discussion

The objective of the present study was to characterize the pharmacological actions of RS 39604. The data obtained suggest that RS 39604 is a high affinity, competitive and selective 5-HT$_4$ receptor antagonist *in vitro* and, as such, is a useful addition to the existing 5-HT$_4$ receptor antagonists. More importantly, the oral activity and long duration of action of RS 39604 suggests that this compound may be the preferable pharmacological probe to use for investigating the physiological and pathophysiological significance of 5-HT$_4$ receptors *in vivo*.

In vitro *pharmacology of RS 39604*

Radioligand binding studies showed that RS 39604 binds with high affinity (pK_i = 9.1) to a single population of 5-HT$_4$ receptors in the guinea-pig striatum. This finding was confirmed in functional studies in which affinities of 9.3 and 9.1 were obtained for RS 39604 in the rat isolated oesophagus and guinea-pig ileal mucosa, respectively. In both the aforementioned preparations, RS 39604 produced parallel, dextral shifts of the concentration-effect curve to the agonist without altering the maximum response; consistent with a competitive mode of interaction. This is in contrast to SB 204070 (Wardle *et al.*, 1994; Baxter *et al.*, 1994) and GR 125487 (Gale *et al.*, 1994b), which behave as unsurmountable antagonists. The unsurmountable nature of SB 204070 has been attributed to its slow dissociation off the receptor leading to 'pseudoirreversible inhibition' (Wardle *et al.*, 1994; Baxter *et al.*, 1994). Accordingly, the lower affinity and faster off-rate of RS 39604 may enable it to attain rapid equilibrium with the agonist thereby facilitating surmountable antagonism. RS 39604 also displayed a high degree of selectivity for 5-HT$_4$ receptors as it possessed a low affinity (pK_i < 6.5) for the vast majority of other receptors studied. RS 39604 possessed moderate affinity (pK_i = 7.8) for the σ_2 site. The relevance of this finding is unclear as the functional significance of the σ_2 receptor has yet to be established (Walker *et al.*, 1990). RS 39604 thus fulfils several criteria (high affinity, selectivity and competitive) expected for an ideal antagonist and can, therefore, be considered an additional probe for further defining the 5-HT$_4$ receptor.

In vivo *pharmacology of RS 39604*

5-HT-induced tachycardia in the anaesthetized micropig is mediated exclusively by 5-HT$_4$ receptors (Villalon *et al.*, 1992). In this preparation, RS 39604, administered by the intravenous route, produced dose-dependent inhibition of 5-HT-induced tachycardia and, in this regard, was as potent as SB 204070, despite possessing a much lower affinity than the latter compound for 5-HT$_4$ receptors. This apparent discrepancy may perhaps be explained by differences in metabolic clearance of the two compounds (see below). RS 39604 was also effective when administered intraduodenally although its potency by this route was approximately 50 fold lower than that by the intravenous route. SB 204070, however, was inactive by the intraduodenal route even at doses up to 3 mg kg^{-1}, suggesting either poor absorbtion or extensive first-pass metabolism of this drug. An unexpected finding in the present study was that SB 204070, when administered intravenously as a single bolus maximal dose, evoked a positive chronotropic effect. This response to SB 204070 appears to be mediated, at least in part, by 5-HT$_4$ receptors in as much as it could be partially reversed by DAU 6285. This finding is difficult to reconcile with previous *in vitro* studies that have failed to reveal a partial agonist effect of SB 204070 even in preparations possessing a high receptor reserve of 5-HT$_4$ receptors such as the guinea-pig distal colon (Wardle *et al.*, 1994). The precise explanation for this disparity has yet to be resolved. The apparent partial agonist effect of SB 204070 precluded the accurate determination of the duration of action of this compound. When one compares equi-effective doses (30 μg kg^{-1}, i.v.) of RS 39604 and SB 204070 at 90 min post-dose (at which time the intrinsic agonist effect of SB 204070 had faded completely), the tachycardic response to 5-HT was inhibited by 80% and 35%, respectively. In a similar model, it was reported that GR 125487 had a duration of action of 75 min (Gale *et al.*, 1994c) whereas the present study has shown that RS 39604 has a duration of action of greater than 6 h. These data suggest, therefore, that the antagonistic effect of RS 39604 is more long-lasting than that of SB 204070 and GR 125487. The most likely explanation for this finding is that RS 39604, in contrast to SB 204070 and GR 125487, lacks the metabolically labile ester functionally (Clark *et al.*, 1994).

We have shown previously that 5-HTP-induced diarrhoea in mice is mediated by 5-HT$_4$ receptors as it can be antagonized by selective 5-HT$_4$ receptor antagonists such as DAU 6285, GR 113808 and SB 204070 (Hegde *et al.*, 1994b). In the present study, we showed that RS 39604, administered intraperitoneally or orally, produced significant inhibition of 5-HTP-induced diarrhoea. A non-specific effect of RS 39604 may be discounted as this compound failed to inhibit prostaglandin-induced diarrhoea. In as much as 5-HT$_4$ receptor activation stimulates electrolyte secretion in the human gut (Burleigh & Borman, 1993; Budhoo & Kellum, 1994), it is plausible that compounds, such as RS 39604, may be useful in combating the diarrhoea associated with carcinoid (Feldman, 1987) and irritable bowel syndrome (Camilleri & Prather, 1992). The p.o./i.p. potency ratio of RS 39604 was calculated to be approximately 14, suggesting only modest absorption of this compound from the gastrointestinal tract. The intraperitoneal potency of RS 39604 was approximately 25 fold lower than that reported previously for SB 204070 (Hegde *et al.*, 1994b). However, the maximal inhibition produced by RS 39604 was approximately 70% which compares with 30% for SB 204070. The precise reason for the lower maximal inhibitory effect of SB 204070 is unclear at present. The lack of activity of SB 204070 by the oral route may also be a reflection of its low oral bioavailibity in mice.

5-HT-induced contraction of the proximal colon *in vitro* (Elswood *et al.*, 1991) or *in vivo* (Hegde *et al.*, 1994a) is mediated via activation of 5-HT$_4$ receptors. Both RS 39604 and SB 204070 potently antagonized the contractile responses to 5-HT in a surmountable and unsurmountable manner, respectively, consistent with the behaviour of these compounds *in vitro*. A comparison of the dose-ratios would suggest that SB 204070 is at least 3 fold more potent than RS 39604. RS 39604 was also effective by the intraduodenal route although it was approximately 10 fold less potent.

Previous studies have shown that inhibition of colorectal distension-induced pressor response in conscious rats is a reliable predictor of visceral antinociceptive activity and response to clinically effective drugs such as morphine and clonidine (Ness & Gebhart, 1988). Furthermore, a role of 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$ receptors in the modulation of visceral nociception has been demonstrated in this animal model (Danzebrink & Gebhart, 1991). The present study has shown that RS 39604, at doses up to 1 mg kg^{-1}, i.v., had no effect on colorectal distension-induced pressor responses. Although data pertaining to the penetration of RS 39604 across the blood-brain-barrier is unavailable at present, the results do not support a role of 5-HT$_4$ receptors in modulation of visceral antinociception. Such as conjecture may imply that selective 5-HT$_4$ antagonists would be ineffective in ameliorating the pain component of irritable bowel syndrome (Camilleri & Prather, 1992).

In summary, the findings of this study suggest that RS 39604 is a selective, orally active and long-acting 5-HT$_4$ receptor antagonist. Consequently, it may represent a significant improvement over some of the existing 5-HT$_4$ antagonists in assessing the physiological role of the 5-HT$_4$ receptor *in vivo*.

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