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GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor

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1 The 5-HT_4 receptor has only recently been identified but has yet to be cloned. This paper describes the pharmacology of a potent and selective 5-HT_4 receptor antagonist, GR113808, which will be useful in the further characterization of this receptor.

2 On the guinea-pig ascending colon, GR113808 (1 nM–0.1 μM) behaved as an antagonist of 5-hydroxytryptamine (5-HT)-induced contraction, producing rightward displacements of the concentration-effect curve to 5-HT and a concentration-related depression of the maximum effect. However, the compound had no effect on cholecystokinin (CCK-8)-induced contraction in concentrations up to 1 μM.

3 In the guinea-pig colon preparation, onset and offset of the antagonism by GR113808 of 5-HT-induced contraction was examined. Incubation of the tissues for either 15 min, 30 min or 60 min produced similar rightward displacements of the concentration-effect curves to 5-HT, with no increase in the degree of depression of the maxima with increasing time of incubation. Experiments examining offset of antagonism (0.01 μM) demonstrated that washout for 30 min was required to reverse fully the effects of the antagonist.

4 Potency estimates in the colon for GR113808 were made by determining approximate pA_2 values (30 min) using the Gaddum equation. The values obtained were 9.2, 9.7 and 9.2 when tested against the agonists 5-HT, 5-methoxytryptamine and R,S-zacopride respectively.

5 On the carbachol-contracted tunica muscularis mucosae preparation of the rat thoracic oesophagus, GR113808 behaved as an antagonist of 5-HT-induced relaxation, producing no reduction in maximum response. Analysis of these data yielded a pA_2 of 9.3. GR113808 also antagonised the relaxant effects of 5-methoxytryptamine ($pA_2 = 9.0$) and **R,S**-zacopride ($pA_2 = 9.4$). The compound had no effect on isoprenaline-induced relaxation of the carbachol-contracted oesophagus at a concentration of 1 μM.

6 In tests of selectivity, GR113808 had only low affinity for 5-HT_3 receptors ($pK_i = 6.0$) and had no functional activity at either 5-HT_2 or 5-HT_1-like receptors on vascular smooth muscle preparations. In a range of binding assays, GR113808 was shown to have no appreciable affinity for any other receptor type investigated.

7 In the anaesthetized piglet, GR113808 was a potent antagonist of 5-methoxytryptamine-induced tachycardia (mean $DR_{10} = 97.2\ \mu g\ kg^{-1}\ h^{-1}$). The compound was ineffective against isoprenaline-induced tachycardia.

8 The present results are discussed in comparison with those for existing antagonists at the 5-HT_4 receptor. The results of this study indicate that GR113808 will be a valuable antagonist for studying 5-HT_4 receptor mechanisms *in vitro* and *in vivo* and validate its use as a radioligand for determining 5-HT_4 receptor distribution.

Keywords: 5-HT; 5-HT_4 receptor; 5-HT_4-receptor antagonist; GR113808

Introduction

A new class of 5-hydroxytryptamine (5-HT) receptor termed 5-HT_4, which is positively coupled to adenylyl cyclase, has been identified in various isolated tissue preparations. The responses to 5-HT mediated by this receptor include, stimulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in neurones of mouse colliculi and guinea-pig hippocampus (Dumuis *et al.*, 1989; Bockaert *et al.*, 1990), neuronally-mediated contraction in guinea-pig ileum (Eglen *et al.*, 1990) and colon (Elswood *et al.*, 1991), relaxation of rat oesophagus (Baxter *et al.*, 1991; Reeves *et al.*, 1991) and a positive inotropic response in human and porcine atria (Kaumann, 1990; Kaumann *et al.*, 1990). In addition, the 5-HT_4 receptor has been identified *in vivo* mediating an increase in heart rate (Villalon *et al.*, 1990). In each of these preparations, high concentrations of tropisetron (ICS205-930), much larger than those required for 5-HT_3 receptor antagonism, inhibited the effect of selective agonists whereas other antagonists selective for 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors were without effect.

Until recently, tropisetron was the sole antagonist capable of blocking the effects of 5-HT at the 5-HT_4 receptor, albeit weakly with an affinity constant at the receptor of between 6.0 and 6.5 (Dumuis *et al.*, 1989; Baxter *et al.*, 1991; Elswood *et al.*, 1991). However, this compound has the disadvantages of high affinity for 5-HT_3 receptors and, at high concentrations, ion channel blocking activity (Richardson *et al.*, 1985; Scholtysik *et al.*, 1988). Two novel compounds SDZ205-557 and DAU 6285, have now been described for which increased 5-HT_4 receptor affinity is claimed with a concomitant reductin in affinity for the 5-HT_3 receptor (Buchheit *et al.*, 1992, Schiavone *et al.*, 1992). However, the affinities of these compounds for the 5-HT_4 receptor are relatively low, with pA_2 values of between 6.5 and 7.4. Furthermore, since their affinity for 5-HT_3 receptors has been assessed largely in guinea-pig tissues, a species in which 5-HT_3 receptor antagonists tend to have lower affinity than elsewhere, it is likely that their selectivities have been overestimated (Eglen *et al.*,

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1993). In this study we describe the pharmacology of GR 113808 (see chemical structure shown in Figure 1), a novel, selective antagonist with high-affinity for the 5-HT$_4$ receptor.

Methods

Guinea-pig ascending colon

Female Dunkin-Hartley guinea-pigs (300–400 g) were killed by cervical dislocation and the most proximal portion of the colon (a 10 cm segment, starting 1 cm from the caecum) was removed. Segments were then prepared as described by Elswood *et al.* (1991). Briefly, the colon was divided into 3 cm segments and opened longitudinally. Faecal matter was removed and the mucosa dissected away. The muscle strip was set up in the longitudinal plane in modified Krebs solution of the following composition (mM): NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 1.3 and glucose 11.1 also containing methysergide (1 μM) and ondansetron (10 μM) to block 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ receptors. The modified Krebs solution was maintained at 32°C and bubbled with 95% O_2/5% CO_2. Tissues were allowed to equilibrate for 40 min prior to dosing with an agonist. Responses were measured isometrically.

Agonist concentration-effect curves were constructed by use of sequential dosing, leaving 10 min between doses. The agonist was left in contact with the tissue until the maximum effect was reached, or if there was no response for 2 min, then the tissues were washed. Thirty minutes were left between concentration-effect curves. Antagonists were incubated for 30 min before repeating the agonist concentration-effect curve.

Three agonist concentration-effect curves were constructed with each tissue. Preliminary experiments established that the second and third concentration-effect curves were superimposable. Therefore, in further experiments the second curve was used as a control and the third curve as the test curve in the presence of the antagonist.

As a control for specificity, the effect of the antagonist on concentration-effect curves to cholecystokinin (CCK-8) was investigated by use of a protocol similar to that outlined above. The responses to CCK-8, like those to 5-HT in this preparation, are cholinergically-mediated (see Elswood *et al.*, 1991).

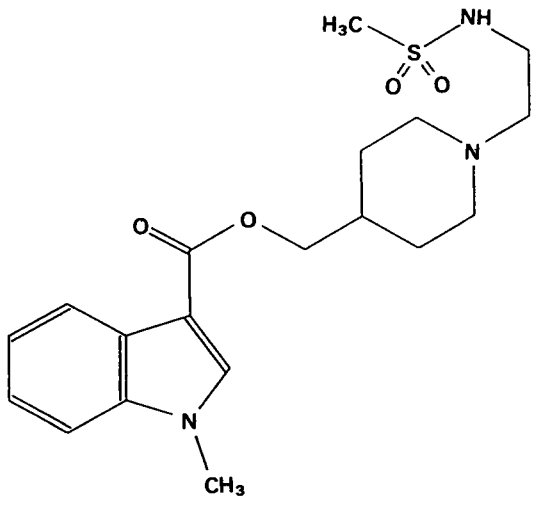

Figure 1 The structure of GR113808 ([1-[2-methylsulphonyl)amino] ethyl]-4-piperidinyl]methyl 1-methyl-IH-indole-3-carboxylate).

Investigation of onset and offset of antagonism due to GR113808

In the colon preparation, experiments were performed to determine the effect of increasing incubation time on the magnitude of antagonism produced with GR113808. Concentration-effect curves were constructed to 5-HT and then repeated following incubation with GR113808 (10.0 nM) for either 15, 30 or 60 min.

In addition, experiments were performed to determine the effect of increasing the washout time of GR113808 following tissue incubation. Concentration-effect curves were constructed to 5-HT and then tissues were incubated for 30 min with GR113808 (10.0 nM).Tissues were then washed every 5 min for either 15, 30 or 60 min and then a second concentration-effect curve to 5-HT was constructed.

Rat thoracic oesophageal muscularis mucosae

Female Wistar rats (120–180 g) were killed by cervical dislocation, the thorax was opened and a central 2 cm portion of the oesophagus removed. The oesophageal segments were prepared as described by Baxter *et al.* (1991). Briefly, the external muscularis propria, containing the outer longitudinal and circular muscle layers of the oesophagus, was carefully dissected in order to isolate the smooth muscle of the tunica muscularis mucosae. The preparations were suspended longitudinally under an initial tension of approximately 0.5 g in modified Krebs solution (composition as before) at 32°C and gassed with 95% O_2/5% CO_2. This solution routinely contained indomethacin (3 μM) and ketanserin (1 μM) to inhibit prostanoid formation and block 5-HT$_2$ receptors respectively. In addition, 3-isobuty-1-methyl-xanthine (IBMX) (3 μM) was included to prevent the breakdown of cyclic AMP and so maximize responses mediated through adenylyl cyclase (Reeves *et al.*, 1991). Responses were measured isometrically.

The oesophageal preparations were contracted by addition of a submaximal concentration of carbachol (1 μM) to the bathing solution. Upon establishment of a stable contraction, a cumulative concentration-effect curve for relaxations to an agonist was constructed. Following the construction of the control curve, the tissues were washed with fresh modified Krebs solution and allowed to recover for 1 h before recontracting with carbachol. Once the preparations had reattained their stable state of contraction to carbachol, the antagonist was added, incubated with the tissue for 30 min and the agonist re-added in a cumulative manner as before.

As a control for specificity, the effect of the antagonist on cumulative concentration-effect curves to isoprenaline was investigated using a protocol similar to the one outlined above on preparations precontracted with carbachol.

Anaesthetized piglet

Anaesthesia was induced in neonatal (10–14 days postpartum) Large White pigs of either sex (4.0–6.0 kg) with 5% isoflurane carried in a mixture of nitrous oxide (2.0 l min^{-1}) and oxygen (1.0 l min^{-1}). Anaesthesia was maintained with a bolus of sodium pentobarbitone (20 mg kg^{-1}, i.v.) followed by intravenous infusion (5 mg kg^{-1} h^{-1}). The animals were artificially respired with air via a tracheal cannula; the stroke volume of the respiration pump was adjusted to keep the arterial blood Po_2, Pco_2 and pH within normal limits (Po_2 = 70–110 mmHg, Pco_2 = 35–55 mmHg, pH = 7.35–7.45). A carotid artery was cannulated to allow measurement of arterial blood pressure and instantaneous heart rate was derived electronically from the signal. Both parameters were displayed continuously on a Devices MX8 pen recorder. Jugular veins were cannulated bilaterally for administration of drugs.

All experiments were carried out in the presence of ondansetron (0.3 mg kg^{-1}, i.v.; given every hour) and ketanserin (1.0 mg kg^{-1}, i.v.; single dose). Cumulative dose-effect curves to 5-methyoxytryptamine (5-MeOT, a 5-HT$_4$ receptor agon-

ist; Villalon *et al.*, 1990) were constructed, leaving 30 min between successive curves.

In antagonist studies, two control dose-effect curves to 5-MeOT were constructed. This was followed by continuous intravenous infusion of the antagonist. After 20 min infusion, a further dose-effect curve to 5-MeOT was constructed.

As a control for specificity, the effect of intravenous infusion of the antagonist on cumulative dose-effect curves to isoprenaline, constructed as described above, was investigated.

Assessment of activity at 5-HT receptors

The activity of GR113808 at vascular 5-HT_2 receptors was assessed by the method of Apperley *et al.* (1976). Briefly, spiral strips were cut from thoracic aortae of New Zealand White rabbits of either sex (2–3 kg). The tissues were suspended in modified Krebs solution (composition as before), maintained at 37°C and bubbled with 95% O_2/5% CO_2. Cumulative concentration-effect curves were constructed to 5-HT (50 nM–50 μM). GR113808 was examined both as a potential agonist (10 nM–50 μM) and also as an antagonist (50 μM) against 5-HT concentration-effect curves.

The activity of GR113808 at the 5-HT_1-like receptor mediating contraction in the dog saphenous vein (Humphrey *et al.*, 1988) and at the 5-HT_1-like receptor mediating relaxation in the porcine vena cava (Sumner *et al.*, 1989) was assessed. Briefly, spiral strips were cut from beagle isolated saphenous vein and rings were cut from the vena cava of neonatal pigs. Preparations were suspended in modified Krebs solution (composition as before), maintained at 37°C and bubbled with 95% O_2/5% CO_2. Preparations of vena cava were precontracted with the thromboxane A_2-mimetic, U-46619 (10 nM). Cumulative concentration-effect curves were constructed to 5-HT (1 nM–10 μM). GR113808 was examined both as a potential agonist (10 nM–50 μM) and also as an antagonist (50 μM) against 5-HT concentration-effect curves.

Specificity data

Further analysis of the specificity and selectivity of GR 113808 was assessed in a range of binding assays performed by Battelle-Europe, Geneva. All tissues were from the rat unless otherwise stated. The following assays were used, the description of the assay being in the form of receptor, ligand, tissue: adenosine A_1, [^{3}H]-DPCPX, cerebral cortex; adenosine A_2, [^{3}H]-CGS-21680, striatum; α_1-adrenoceptor, [^{3}H]-prazosin, whole brain minus cerebellum; α_2-adrenoceptor, [^{3}H]-idazoxan, cerebral cortex; β_1-adrenoceptor, [^{3}H]-CGP-26505, cerebral cortex; β_2-adrenoceptor, [^{125}I]-iodocyanopindolol, guinea-pig lung; dopamine D_1, [^{3}H]-SCH-23390, striatum; dopamine D_2, [^{3}H]-YM-091512, striatum; $GABA_A$, [^{3}H]-muscimol, whole brain; $GABA_B$; [^{3}H]-baclofen, cerebellum; 5-HT_1-like, [^{3}H]-5-HT, cerebral cortex; 5-HT_{1C}, [^{3}H]-mesulergine, porcine choroid plexus; 5-HT_3, [^{3}H]-GR65630, cerebral cortex; muscarinic M_1 cholinoceptor, [^{3}H]-pirenzipine, cerebral cortex; muscarinic M_2 cholinoceptor, [^{3}H]-NMS, heart; muscarinic M_3 cholinoceptor, [^{3}H]-DAMP, pancreas; nicotinic cholinoceptor, [^{3}H]-NMCl, cerebral cortex; histamine H_1, [^{3}H]-mepyramine, cerebral cortex; histamine H_3, [^{3}H]-N-α-methylhistamine, cerebral cortex; NMDA [^{3}H]-CGS-19755, whole brain minus cerebellum; μ-opioid, [^{3}H]-CTOP, whole brain minus cerebellum; κ-opioid, [^{3}H]-U-69593, guinea-pig cerebral cortex; bradykinin, [^{3}H]-bradykinin, guinea-pig ileum; CCK_A, [^{3}H]-L 364718, pancreas; CCK_B, [^{3}H]-L365,260, guinea-pig whole brain minus cerebellum; neurokinin NK_1, [^{3}H]-substance P, whole brain minus cerebellum; neurokinin NK_2, [^{3}H]-neurokinin A, colon; neurokinin NK_3, [^{3}H]-senktide, cerebral cortex; CGRP, [^{125}I]-CGRP, hypothalamus; neuropeptide Y, [^{125}I]-neuropeptide Y, hippocampus; bombesin, [^{125}I]-bombesin, whole brain minus cerebellum; endothelin, [^{125}I]-endothelin, heart; somatostatin, [^{125}I]-somatostatin, cerebral cortex; vasopressin V_1, [^{3}H]-vasopressin, liver; vasopressin V_2, [^{3}H]-vasopressin, kidney; vasoactive intestinal polypeptide, [^{125}I]-VIP, cerebral cortex; galanin, [^{125}I]-galanin, cerebral cortex; benzodiazepine (central), [^{3}H]-flunitrazepam, cerebral cortex; glycine (strychnine insensitive), [^{3}H]-5,7-DCKA, cerebral cortex. Values for pK_i are expressed as the mean value from 3 observations (see Battelle Handbook, 7, Route de Drize, CH-1227 Carouge, Switzerland).

Analytical methods and statistical analysis

All results were expressed as either geometric means with 95% confidence limits or arithmetic means ± s.e.mean of n observations and were compared by Student's paired t test; a P value of <0.05 being considered significant. Concentration-ratios were determined by comparison of the EC_{50} values between control and test curves. EC_{50} values were calculated at the 50% level of control curves to each agonist. pA_2 estimates were determined for GR113808 against 5-HT and **R,S**-zacopride by comparison of the responses at the EC_{50} level for each agonist. In the colon preparation, an apparent pK_B for GR113808 was determined, against 5-methoxytryptamine, at a concentration of 10.0 nM, using the Gaddum equation (Gaddum, 1957). *In vivo*, DR_{10} values were calculated graphically from plots of agonist dose-ratio against dose of antagonist.

Compounds

The following compounds were obtained from commercial sources: 5-HT, 5-MeOT, indomethacin, IBMX and isoprenaline (Sigma Chemical Co), ketanserin (Janssen Pharmaceutica), methysergide (Sandoz) and cholecystokinin octapeptide (CCK-8, Research Plus Inc.). Compounds synthesized by Glaxo Group Research Ltd. were: **R,S**-zacopride, ondansetron, [^{3}H]-GR65630 (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone), U-46619 (9,11-dideoxy methanoepoxy-9d,11α-prostaglandin $F_{2\alpha}$) and GR113808 (([1-[2-methylsulphonyl)amino]ethyl]-4-piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate).

All compounds were solubilized in distilled water except indomethacin (stock solution in 10% w/v $NaHCO_3$ with subsequent dilution in distilled water). Solutions of isoprenaline always contained ascorbic acid (10 μM) as an antioxidant.

Results

Guinea-pig ascending colon

5-HT (3 nM–1 μM), 5-MeOT (30 nM–10 μM) and **R,S**-zacopride (0.1 μM–10 μM) elicited concentration-dependent contractions of the preparations with EC_{50} values of 28.3 (20.5–39.8) nM ($n = 16$), 0.77 (0.35–1.72) μM ($n = 15$) and 0.37 (0.31–0.43) μM ($n = 15$) respectively. Compared to 5-HT, 5-MeOT and **R,S**-zacopride behaved as partial agonists, producing maximum contractions which were 80 ± 6% and 52 ± 4% (at 3 μM) respectively of that to 5-HT. Addition of GR113808 to the bathing medium had no effect on the colon preparation *per se*. In the presence of increasing concentrations of GR113808, the concentration-effect curves to 5-HT, 5-MeOT and **R,S**-zacopride were displaced to the right in a concentration-dependent manner (Figure 2). There was a concentration-related depression of the maximum response. At a concentration of 100 nM GR113808, the maximum effect of 5-HT was reduced to 71.0 ± 6.8% of control values, with 5-MeOT the maximum was reduced to 55.0 ± 10.0% and with **R,S**-zacopride to 62.0 ± 15.0%. By comparison of the responses at the original EC_{50} level for each agonist, Schild analysis yielded pA_2 estimates of: 9.2 ± 0.2 (Schild slope of 1.1 (0.8–1.3)) against 5-HT and 9.2 ± 0.2 (Schild slope of 0.9 (0.6–1.1)) against **R,S**-zacopride. Since the maximum response to 5-MeOT was most affected, the affinity

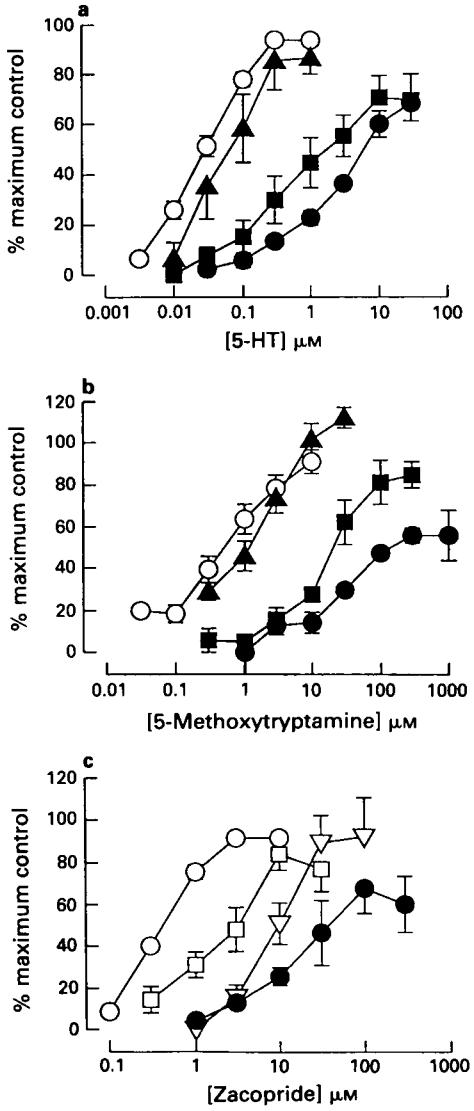

Figure 2 The antagonist effect of GR113808 on the contractile effects of 5-HT in the guinea-pig proximal colon. Curves represent concentration-effect curves to (a) 5-HT, (b) 5-methoxytryptamine and (c) R,S-zacopride in the absence (○) and presence of GR113808 1.0 nM (▲), 3.0 nM (□), 10.0 nM (■), 30.0 nM (▽) and 100.0 nM (●). Results are expressed as means ± s.e.mean, $n = 4-7$.

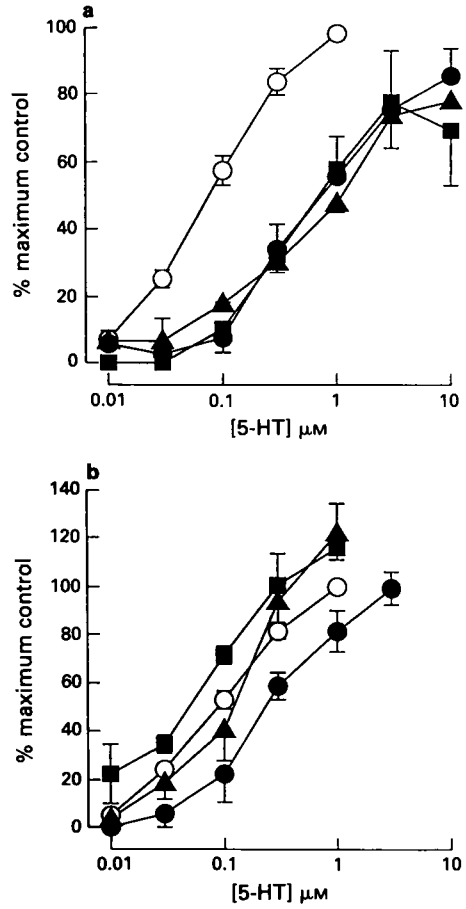

Figure 3 The effect of (a) increasing antagonist equilibration time and (b) increasing antagonist washout of GR113808 (10.0 nM) in the guinea-pig proximal colon. In (a), curves represent concentration-effect curves to 5-HT in the absence (○) and presence of GR113808 (10.0 nM) following tissue incubation for either 15 min (●), 30 min (▲) or 60 min (■). In (b), curves represent concentration-effect curves to 5-HT in the absence (○) of GR113808 and after pretreatment for 30 min with GR113808 (10.0 nM) followed by washout for either 15 min (●), 30 min (▲) or 60 min (■). Results are expressed as means ± s.e.mean, $n = 3-4$.

could only be estimated from a single antagonist concentration (10.0 nM). Use of the Gaddum equation yielded an apparent pK_B of 9.7 ± 0.2.

Cholecystokinin (CCK-8) (3 nM–0.3 μM) elicited concentration-dependent contractions of the colon preparation with an EC_{50} value of 5.5 (3.5–8.7) nM ($n = 6$). Incubation of the tissues with 1 μM GR113808 had no significant effect on the concentration-effect curves to CCK-8 (concentration-ratio = 1.9 (1.5–2.5)).

Investigation of onset and offset of antagonism due to GR113808

Onset 5-HT (10 nM–1 μM) elicited concentration-dependent contractions of the colon preparation with an EC_{50} value of 80.0 (60.0–110.0) nM ($n = 9$). Incubation with GR113808 (10.0 nM) for either 15, 30 or 60 min displaced 5-HT concentration-effect curves to the right, producing curves with EC_{50} values of 0.78 (0.46–1.33) μM ($n = 3$), 1.22 (0.79–1.88) μM ($n = 3$) and 0.88 (0.07–12.70) μM ($n = 3$) respectively (Figure 3a). The EC_{50} values calculated in the presence of antagonist were not signficantly different from one another ($P = 1.0$ comparing 15 min with 30 min, $P = 0.7$ comparing 15 min with 60 min). Furthermore, there was no increase in the depression of the maximum responses with increasing incubation time (see Figure 3).

Offset 5-HT (10 nM–1 μM) elicited concentration-dependent contractions of the colon preparation with an EC_{50} value of 90.0 (70.0–120.0) nM ($n = 16$). Incubation of the tissues for 30 min with GR113808 (10.0 nM) followed by repeated washout for either 15, 30 or 60 min and subsequent construction of concentration-effect curves to 5-HT yielded concentration-dependent contractile responses (Figure 3b). After washout for 15 min the EC_{50} value for the 5-HT concentration-effect curve was 0.21 (0.10–0.43) μM ($n = 4$). After washout for 30 min the EC_{50} value was 0.11 (0.04–0.29) μM ($n = 4$) and after washout for 60 min the EC_{50} was 0.11 (0.4–0.7) μM ($n = 4$). Only the EC_{50} value following washout for 15 min was significantly different from control values ($P < 0.01$).

Rat thoracic oesophageal muscularis mucosae

5-HT (1 nM–1 μM), 5-MeOT (1 nM–3 μM) and R,S-zacopride (1 nM–3 μM) elicited concentration-dependent relaxations of the precontracted rat thoracic oesophagus with EC_{50}

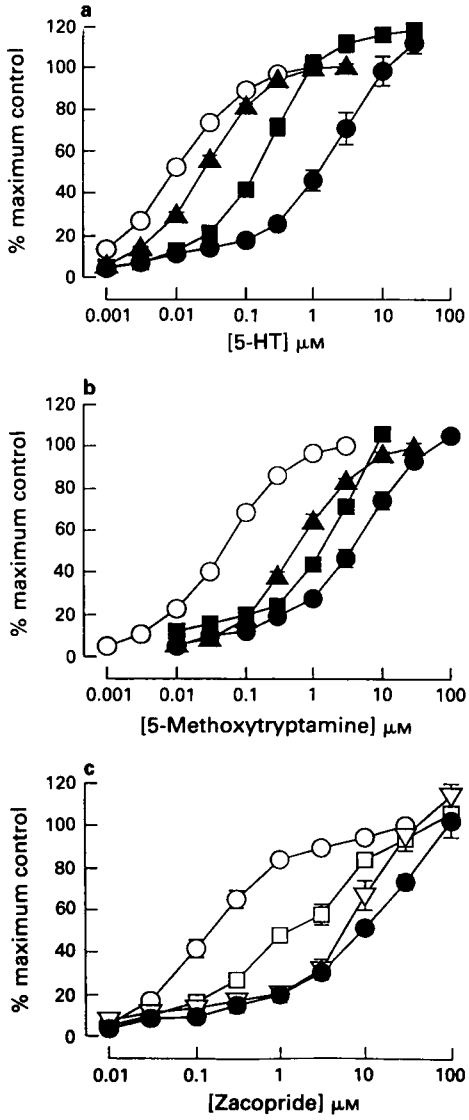

Figure 4 The antagonist effect of GR113808 on the relaxant response to (a) 5-HT, (b)-methoxytryptamine (5-MeOT) and (c) **R,S**-zacopride in the rat thoracic oesophagus. Curves represent concentration-effect curves to (a) 5-HT and (b) 5-MeOT in the absence (○) and presence of GR113808 1.0 nM (▲), 3.0 nM (□), 10.0 nM (■), 30.0 nM (▽) and 100.0 nM (●). Results are expressed as means ± s.e.mean, $n = 4-7$.

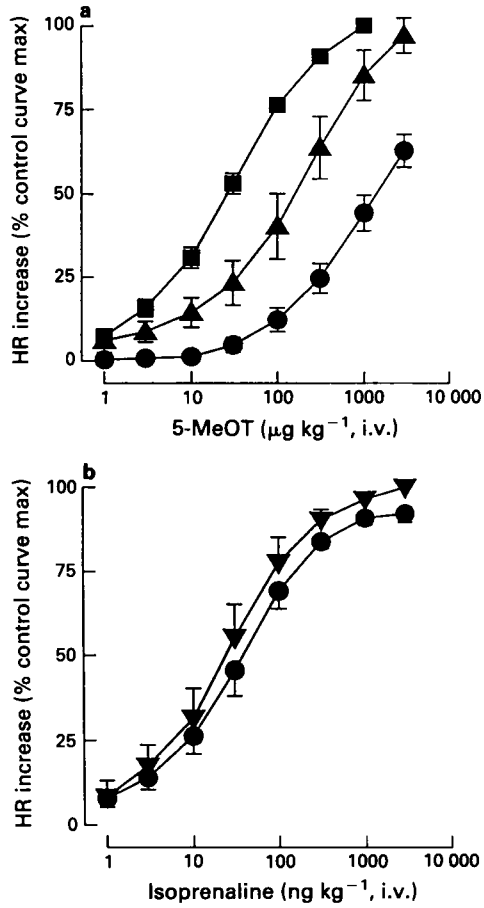

Figure 5 The antagonist effect of intravenous infusion of GR113808 on the increase in heart rate elicited by cumulative bolus intravenous administration of (a) 5-methoxytryptamine (5-MeOT) and (b) isoprenaline. Curves represent (a) 5-MeOT control (■) and dose-effect curves to 5-MeOT following 20 min infusion of GR113808 at 0.1 mg kg^{-1} h^{-1} (▲) and 0.5 mg kg^{-1} h^{-1} (●) or (b) isoprenaline dose-effect curves in the absence (▼) of GR113808 and following 20 min infusion of GR113808 at 0.5 mg kg^{-1} h^{-1} (●). Results are expressed as means ± s.e.mean, $n = 4$.

values of 9.0 (7.0–11.0) nM ($n = 12$), 47 (33–67) nM ($n = 10$) and 0.12 (0.07–0.20) μM ($n = 9$) respectively. Compared to 5-HT, 5-MeOT and **R,S**-zacopride behaved as full agonists. Addition of GR113808 to the bathing medium did not cause relaxation of the oesophagus preparation. In a number of tissues a small increase in the magnitude of the carbachol-induced contraction was observed following addition of the antagonist. In the presence of increasing concentrations of GR113808, the concentration-effect curves to 5-HT, 5-MeOT and **R,S**-zacopride were displaced to the right in a concentration-dependent manner (Figure 4). The maximum responses to all agonists was not affected by incubation with the antagonist. By comparing responses at the EC_{50} level for each agonist, Schild analysis yielded pA_2 estimates of 9.3 ± 0.1 (Schild slope of 0.9 (0.8–1.0)) against 5-HT, 9.0 ± 0.1 (Schild slope of 0.9 (0.8–1.1)) against 5-MeOT and 9.4 ± 0.2 (Schild slope of 0.8 (0.6–1.0)) against **R,S**-zacopride.

Isoprenaline (0.1 nM–0.3 μM) elicited concentration-dependent relaxations of the oesophageal preparations with an EC_{50} value of 1.27 (0.79–2.06) nM ($n = 6$). Incubation of the tissues with 1 μM GR113808 had no significant effect on the concentration-effect curves to isoprenaline (concentration ratio = 1.47 (0.96–2.27)).

Anaesthetized piglet

Intravenous bolus administration of 5-MeOT elicited dose-dependent increases in heart rate in the anaesthetized piglet, with an ED_{50} value of 25.3 (18.4–34.8) μg kg^{-1} ($n = 8$). In preliminary experiments, at least three dose-effect curves were shown to be reproducible (agonist dose-ratios between curves 1 and 2 and between 1 and 3 were 1.1 and 0.8 respectively). Following intravenous infusion of GR113808 at 0.1 mg kg^{-1} h^{-1} and 0.5 mg kg^{-1} h^{-1} for 20 min, the dose-effect curves to 5-MeOT were displaced to the right in a parallel fashion giving rise to agonist dose-ratios of 6.4 (1.9–21.6, $n = 4$) and 53.5 (15.9–180.0, $n = 4$) respectively (Figure 5a). The *in vivo* potency of the antagonist, expressed as a mean DR_{10} value calculated from both doses of antagonist, was 97.2 (53.4–177.0) μg kg^{-1} h^{-1}.

Intravenous bolus administration of isoprenaline elicited dose-dependent increases in heart rate in the piglet, with an ED_{50} value of 24.1 (3.8–153.8) ng kg^{-1} ($n = 3$). Successive dose-effect curves were shown to be repeatable (agonist dose-ratios between curves 1 and 2 and between curves 1 and 3 were 1.1. and 1.0 respectively). Following intravenous infu-

Table 1 The affinity of GR113808 for a range of receptor types assessed by binding assays (details in text)

Receptor	*Affinity* (pK_i)	*Receptor*	*Affinity* (pK_i)
Adenosine A_1	<5.0	Bombesin	<5.0
Adenosine A_2	<5.0	Endothelin	<5.0
α_1-Adrenoceptor	<5.0	Somatostatin	<5.0
α_2-Adrenoceptor	<5.0	Vasopressin V_1	<5.0
β_1-Adrenoceptor	5.1	Vasopressin V_2	<5.0
β-Adrenoceptor	<5.0	VIP	<5.0
Dopamine D_1	<5.0	Galanin	<5.0
Dopamine D_2	<5.0	Benzodiazepine	<5.0
$GABA_A$	<5.0	Glycine	<5.0
$GABA_B$	<5.0	μ-Opioid	<5.0
5-HT_1	<5.0	κ-Opioid	<5.0
5-HT_{1C}	<5.0	Bradykinin	<5.0
5-HT_3	6.0	CCK_A	<5.0
M_1 cholinoceptor	5.2	CCK_B	<5.0
M_2 cholinoceptor	<5.0	Neurokinin NK_1	<5.0
M_3 cholinoceptor	<5.0	Neurokinin NK_2	<5.0
Nicotinic cholinoceptor	<5.0	Neurokinin NK_3	<5.0
Histamine H_1	<5.0	CGRP	<5.0
Histamine H_3	<5.0	Neuropeptide Y	<5.0
NMDA	<5.0		

sion of GR113808 at 0.5 mg kg^{-1} h^{-1} for 20 min, the dose-effect curves to isoprenaline were unaffected (Figure 5b).

Selectivity at 5-HT receptors

GR113808 (10 nM–50 μM) did not exhibit any agonist activity at the 5-HT_2 receptor nor at the 5-HT_1-like receptors mediating contraction in the dog saphenous vein or 5-HT receptors mediating relaxation in the porcine vena cava. Furthermore, at 50 μM the compound did not affect contractile responses to 5-HT in either rabbit aorta or dog saphenous vein, nor the relaxant response to 5-HT in the porcine vena cava ($n = 2$). In addition, GR113808 was shown to have only low affinity at the 5-HT_3 receptor (pK_i = 6.0). Furthermore, GR113808 had no measurable affinity (pK_i < 5.0) at any of other 5-HT receptors examined in binding assays (Table 1).

Specificity

GR113808 had no significant binding affinity at the majority of receptor types examined. However, the compound was shown to have measurable affinity at the β_1-adrenoceptor (pK_i = 5.0) and the muscarinic M_1 cholinoceptor (pK_i = 5.2) (Table 1).

Discussion

In this study we have shown GR113808 to be a specific antagonist with high affinity for the 5-HT$_4$ receptor in guinea-pig isolated ascending colon and rat isolated oesophagus. It behaved as a surmountable antagonist in both preparations although some reduction in the maximum responses to agonists was observed in the colon. This effect on the maximum response to 5-HT$_4$ receptor agonists was apparently not attributable to a non-specific effect of GR113808 since responses to CCK-8 and isoprenaline were not affected in the colon and oesophagus preparations respectively.

A possible explanation for the depression of the maximum responses to agonists seen in the presence of GR113808 in the colon, but not in the oesophagus, may relate to a lack of steady-state conditions for the former preparation (for discussion of this topic, see Kenakin, 1987). Thus, the maxima of concentration-effect curves to agonists would be expected to be depressed if either (a) the antagonist dissociates slowly from the receptor or (b) the spare receptor population is low and/or the receptor-response coupling efficiency is poor, as would be so for a partial agonist (see Rang, 1966). Our data show that the offset rate for washout of GR113808 is slow in the colon preparation; after 15 min washing the effect of GR113808 was not fully reversed. However, this probably reflects the effects of diffusion of the drug from the tissue rather than slow dissociation of the antagonist from the receptor *per se*, since GR113808 has been shown to dissociate rapidly (<3 min) in radioligand binding studies using guinea-pig striatal membranes (Grossman *et al.*, 1993). Nevertheless, the fast, phasic response to agonists in the colon, in contrast to the slow response in the oesophagus, dictates that the agonist is only in contact for up to 2 min which might have contributed to the lack of steady-state conditions and apparent lack of competitive antagonism by GR113808 in colon. In addition, there seems to be a low reserve for 5-HT$_4$ receptors in the colon, as judged by the low potency of 5-HT and supported by the partial agonist activity of 5-MeOT and **R,S**-zacopride. This contrasts with the oesophagus where all the agonists tested produced similar maxima and were more active at lower concentrations than in the colon. The observed rise in tone in the precontracted oesophagus preparation, following application of the antagonist, is likely to be a result of antagonism of on-going relaxation produced by the release of endogenous 5-HT elicited by carbachol (Waikar *et al.*, 1993). These findings are entirely consistent with the concept that GR113808 is a competitive antagonist and that the reduction in agonist maxima that it produced in guinea-pig colon relates to a lack of steady-state conditions under the experimental conditions necessarily used for this preparation.

GR113808 is more potent than other 5-HT$_4$ receptor antagonists so far described, DAU 6285 (endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1] oct-3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1 carboxylate hydrochloride) and SDZ205-557 (2-methoxy-4-amino-5-chloro benzoic acid 2-(diethylamino) ethyl ester). These compounds have only modest affinity at the 5-HT$_4$ receptor. DAU 6285 has a pA_2 of between 6.64 and 7.16 in the guinea-pig ileum and human atrium respectively (Schiavone *et al.*, 1992), whereas SDZ205-557 has a slightly higher pA_2 of 7.4 in the guinea-pig ileum (Buchheit *et al.*, 1992) and a pA_2 of 7.3 in the rat oesophagus (Eglen *et al.*, 1993). In addition, *in vivo*, GR113808 was shown to be a very potent antagonist of the tachycardia response elicited by 5-MeOT (DR_{10} = 97.2 μg kg^{-1} h^{-1}); this response has previously been shown to be mediated by the 5-HT$_4$ receptor (Villalon *et al.*, 1990).

To date, tropisetron and DAU 6285 are the only 5-HT$_4$

receptor antagonists described in studies *in vivo* (Villalon *et al.*, 1990; Van Meel *et al.*, 1993). Tropisetron partially blocked the tachycardic effects of indole and benzamide agonists at the 5-HT_4 receptor on porcine heart. However, the compound exhibited only low potency, an intravenous bolus dose of 3 mg kg^{-1} being required to produce less than a 10 fold displacement to the right of the dose-effect curves (Villalon *et al.*, 1990). Similarly, between 1 and 3 mg kg^{-1}, i.v. DAU 6285 produced approximately a 10 fold displacement to the right of a 5-HT dose-effect curve in an anaesthetized pig model (Van Meel *et al.*, 1993). Although we are attempting to compare the effects of bolus doses of tropisetron and DAU 6285 with the effects of an intravenous infusion of GR113808, our data strongly suggests that GR 113808 is a considerably more potent 5-HT_4 receptor antagonist *in vivo*.

GR113808 exhibits a high degree of selectivity and specificity both *in vitro* and *in vivo*. The compound showed approximately 3000-fold selectivity between 5-HT_4 receptors and the other 5-HT receptors investigated. In particular, GR113808 exhibited a pK_i at the 5-HT_3 receptor of only 6.0 which makes the compound considerably more selective than the other 5-HT_4 receptor antagonists DAU 6285 and SDZ 205-557 (Buchheit *et al.*, 1992; Schiavone *et al.*, 1992; Eglen *et al.*, 1993). Furthermore, GR113808 demonstrated a high degree of specificity, showing at least a 10,000 fold specificity between 5-HT_4 receptor and the other non-5-HT receptor types examined.

Thus, GR113808 is a high affinity, selective and specific 5-HT_4 receptor antagonist which has potent activity *in vivo*. This compound therefore represents a useful pharmacological tool for investigating 5-HT_4 receptor-mediated events and for defining the role of this receptor in mammalian physiology and pathophysiology. Furthermore, the pharmacological profile validates its usefulness as a radioligand for the 5-HT_4 receptor (see Grossman *et al.*, 1993).

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References

APPERLEY, E., HUMPHREY, P.P.A. & LEVY, G.P. (1976). Receptors for 5-hydroxytryptamine and noradrenaline in rabbit isolated ear artery and aorta. *Br. J. Pharmacol.*, **58**, 211–221.

BAXTER, G.S., CRAIG, D.A. & CLARKE, D.E. (1991). 5-Hydroxytryptamine$_4$ receptors mediate relaxation of the rat oesophageal tunica muscularis mucosae. *Naunyn-Schmied. Arch. Pharmacol.*, **343**, 439–446.

BOCKAERT, J., SEBBEN, M. & DUMUIS, A. (1990). Pharmacological characterization of 5-hydroxytryptamine$_4$ (5-HT_4) receptors positively coupled to adenylate cyclase in adult guinea-pig hippocampal membranes: Effect of substituted benzamide derivatives. *Mol. Pharmacol.*, **37**, 408–411.

BUCHHEIT, K.-H., GAMSE, R. & PFANNKUCHE, H.-J. (1992). SDZ 205-557, a selective, surmountable antagonist for 5-HT_4 receptors in the isolated guinea-pig ileum. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 387–393.

DUMUIS, A., SEBBEN, M. & BOCKAERT, J. (1989). The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT_4) positively coupled to adenylate cyclase in neurones. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 403–410.

EGLEN, R.M., ALVAREZ, R., JOHNSON, L.G., LEUNG, E. & WONG, E.H.F. (1993). The action of SDZ 205,557 at 5-hydroxytryptamine (5-HT_3 and 5-HT_4) receptors. *Br. J. Pharmacol.*, **108**, 376–382.

EGLEN, R.M., SWANK, S.R., DUBUQUE, R.L. & WHITING, R.L. (1990). Characterization of 5-HT receptors mediating contractions of guinea-pig ileum *in vitro*. *Br. J. Pharmacol.*, **99**, 216P.

ELSWOOD, C.J., BUNCE, K.T. & HUMPHREY, P.P.A. (1991). Identification of 5-HT_4 receptors in guinea-pig ascending colon. *Eur. J. Pharmacol.*, **196**, 149–155.

GADDUM, J.H. (1957). Series of drug antagonism. *Pharmacol. Rev.*, **9**, 211–218.

GROSSMAN, C.J., KILPATRICK, G.J. & BUNCE, K.T. (1993). Development of a radioligand binding assay for the 5-HT_4 receptor: use of a novel antagonist. *Br. J. Pharmacol.*, **109**, 618–624.

HUMPHREY, P.P.A., FENIUK, W., PERREN, M.J., CONNOR, H.E., OXFORD, A.W., COATES, I.H. & BUTINA, D. (1988). GR43175, a selective agonist for the 5-HT_1-like receptor in dog isolated saphenous vein. *Br. J. Pharmacol.*, **94**, 1123–1132.

KAUMANN, A.J. (1990). Piglet sinoatrial 5-HT receptors resemble human atrial 5-HT_4-like receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 619–622.

KAUMANN, A.J., SANDERS, L., BROWN, A.M., MURRAY, K.J. & BROWN, M.J. (1990). A 5-hydroxytryptamine receptor in human atrium. *Br. J. Pharmacol.*, **100**, 879–885.

KENAKIN, T.P. (1987). Kinetics of drug action, Chapter 12. In *Pharmacologic Analysis of Drug-Receptor Interaction*. New York: Raven Press.

RANG, H.P. (1966). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. R. Soc.*, **164**, 488–510.

REEVES, J.J., BUNCE, K.T. & HUMPHREY, P.P.A. (1991). Investigation into the 5-HT receptor mediating smooth muscle relaxation in rat oesophagus. *Br. J. Pharmacol.*, **103**, 1067–1072.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STADLER, P.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

SCHIAVONE, A., GIRALDO, E., GIUDICI, L., TURCONI, M. & SAGRADA, A. (1992). DAU 6285: a novel antagonist at the putative 5-HT_4 receptor. *Life Sci.*, **51**, 583–592.

SCHOLTYSIK, G., IMOTO, Y., YATANI, A. & BROWN, A.M. (1988). 5-Hydroxytryptamine antagonist ICS205-930 blocks cardiac potassium, sodium and calcium currents. *J. Pharmacol. Exp. Ther.*, **245**, 773–778.

SUMNER, M.J., FENIUK, W. & HUMPHREY, P.P.A. (1989). Further characterization of the 5-HT receptor mediating vascular relaxation and elevation of cyclic AMP in porcine isolated vena cava. *Br. J. Pharmacol.*, **97**, 292–300.

VAN MEEL, J.C.A., DIEDEREN, W., HAIGH, R., WIENEN, W., PAIRET, M., TURCONI, M. & DOODS, H.N. (1993). The novel 5-HT_4 receptor antagonist DAU 6285 antagonizes 5-hydroxytryptamine-induced tachycardia in pigs. *Eur. J. Pharmacol.*, **233**, 295–297.

VILLALON, C.M., DENBOER, M.O., HEILIGERS, J.P.C. & SAXENA, P.R. (1990). Mediation of 5-hydroxytryptamine-induced tachycardia in the pig by the putative 5-HT_4 receptor. *Br. J. Pharmacol.*, **100**, 665–667.

WAIKAR, M.V., HEDGE, S.S., FORD, A.P.D.W. & CLARKE, D.E. (1993). Pharmacological analyses of endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1] oct-3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxylate hydrochloride (DAU 6285) at the 5-hydroxytryptamine$_4$ receptor in the tunica muscularis mucosae of rat esophagus and ileum of guinea-pig: role of endogenous 5-hydroxytryptamine. *J. Pharmacol. Exp. Ther.*, **264**, 654–661.

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