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Running title: pharmacological profile of the h5-HT$_{4(d)}$ receptor

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Abstract

1 The recently identified C-terminal splice variant of the human 5-HT$_4$ receptor, the h5-HT$_{4(d)}$ receptor, was stably expressed in a CHO cell line at 493±25 fmol mg^{-1} protein. We analysed its pharmacological properties by measuring binding affinities and 5-HT$_4$ ligand-induced cAMP production.

2 The pharmacological binding profile determined in competition studies with the specific antagonist [^{3}H]-GR113808 revealed a rank order of affinity of 5-HT$_4$ compounds for the h5-HT$_{4(d)}$ receptor that was consistent with those previously reported for other 5-HT$_4$ receptor isoforms.

3 In adenylyl cyclase functional assays, the h5-HT$_{4(d)}$ receptor displayed equipotent coupling for all 5-HT$_4$ agonists tested (EC_{50} in the range of 1 to 6 nM). EC_{50} values were lower than those previously obtained with the 5-HT$_{4(e)}$ receptor stably expressed in CHO cells indicating that the 5-HT$_{4(d)}$ receptor was more efficiently coupled to its effector than the 5-HT$_{4(e)}$ receptor isoform. Moreover, in terms of agonist efficacy (E_{max}), the benzamide derivatives, renzapride displayed full agonist properties at the h5-HT$_{4(d)}$ receptor (same E_{max} as 5-HT) whereas it was previously shown to be a partial agonist at the h5-HT$_{4(e)}$ receptor.

4 A constitutive activity of the h5-HT$_{4(d)}$ receptor was observed in CHO cells in the absence of any 5-HT$_4$ ligand. Surprisingly, two 5-HT$_4$ compounds, SB204070 and RS39604 which are described as highly potent antagonists in various biological models, revealed partial agonist properties at the h5-HT$_{4(d)}$ receptor.

5 We conclude that C-terminal tails of 5-HT$_4$ receptor isoforms may directly influence their functional properties.

Keywords: Human, serotoninergic receptors, 5-HT$_4$ ligands, inverse agonism, G-protein coupled receptors, adenylyl cyclase, benzamides.

Abbreviations:

- 5-HT, 5-hydroxytryptamine
- 5-MeOT, 5-methoxytryptamine
- BIMU1, endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide
- CHO cells, chinese hamster ovary cells
- cisapride, *cis*-4-amino-5-chloro-*N*-[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide
- GPCR, G-protein-coupled receptors
- GR113808, [1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate)
- GR127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'- methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide
- h5-HT$_4$, human 5-HT$_4$ receptor
- ML10302, 2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride
- ML10375, 2-[*cis*-3,5-dimethylpiperidino]ethyl 4-amino-5-chloro-2-methoxybenzoate
- renzapride (BRL 24924), (±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride
- RS23597, 3-(piperidine-1-yl)-propyl-4-amino-5-chloro-2-methoxybenzoate hydrochloride
- RS39604, 1-(5-chloro-2(3,5-dimethoxy)benzyloxy-4-aminophenyl)-3-(N-(methylsulfamido)ethyl-4-piperidyl)propanone
- RS67333, 1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-n-butyl-4-piperidinyl)-1-propanone
- SB204070, 8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride

Introduction

Serotonin [5-hydroxytryptamine, 5-HT] mediates a large diversity of physiological effects both in the central nervous system and the periphery of vertebrates, through a complex mechanism involving at least seven classes of receptor (5-HT$_1$-5-HT$_7$) (Saxena, 1995). Each family of receptors is characterised by distinct molecular and pharmacological properties and subdivided in several receptor subtypes, conferring an additional level of complexity in the system (Gerhardt & Van Heerikhuizen, 1997).

5-HT$_4$ receptors were first characterised by their ability to stimulate cAMP production in mouse colliculi neurones and by the inability of classical 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$ antagonists to inhibit this function (Dumuis *et al.*, 1988). Since that discovery, numerous pharmacological studies have been done in a wide variety of tissues and species indicating that 5-HT$_4$ receptors were highly distributed in the central nervous system (Eglen *et al.*, 1995) and peripheral tissues of vertebrates including alimentary tract, urinary bladder, heart and adrenal gland (Hedge and Eglen, 1996). Despite the existence of a typical 5-HT$_4$ pharmacological profile, heterogeneity in the potency and intrinsic activities of 5-HT$_4$ ligands has been reported in different biological models. For instance, two substituted benzamides, cisapride and renzapride behaved as full and potent agonists in mouse colliculi neurones (Dumuis *et al.*, 1989) but were only partial in guinea pig hippocampal membranes (Bockaert *et al.*, 1990), rat esophagus (Baxter *et al.*, 1991), rat distal colon (Wardle & Sanger, 1993) and human heart atrium (Ouadid *et al.*, 1991). The agonist ML10302 displayed a poor agonistic effect in receptor cloned from human atrium (Blondel *et al.*, 1997) whereas it was full agonist in rat oesophagea (Langlois *et al.*, 1994). Altogether, these observations make it difficult to appreciate the functional potency of a given 5-HT$_4$ ligand and suggest the existence of several 5-HT$_4$ receptor subtypes with distinct intrinsic pharmacological properties.

A confirmation of the presence of multiple 5-HT$_4$ receptors has been made with the molecular identification of five C-terminal splice variants of the human 5-HT$_4$ receptor (h5-HT$_4$) named h5-HT$_{4(a)}$, h5-HT$_{4(b)}$, h5-HT$_{4(c)}$, h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ (Blondel *et al.*, 1997; Claeysen *et al.*, 1997; Van den Wyngaert *et al.*, 1997; Blondel *et al.*, 1998; Claeysen *et al.*, 1999; Mialet *et al.*, 2000). In addition, a novel h5-HT$_4$ splice variant (h5-HT$_{4(hb)}$) with an extra insertion of 14 amino acids in the second extracellular loop has been very recently cloned (Bender *et al.*, 2000). These receptors belong to the family of seven transmembrane domain G-protein coupled family of receptors which activate adenylyl cyclase. Tissue distribution studies revealed some degree of specificity in the pattern of expression of the h5-HT$_4$ receptor isoforms with h5-HT$_{4(a)}$, h5-HT$_{4(b)}$, h5-HT$_{4(c)}$ and h5-HT$_{4(e)}$ receptors being expressed in cardiac atria and brain (Blondel *et al.*, 1998; Mialet *et al.*, 2000). Interestingly, expression of the h5-HT$_{4(d)}$ receptor was only restricted to the gut (Blondel *et al.*, 1998). This distinct spatial distribution may explain the difference in efficacy of 5-HT$_4$ ligands on 5-HT$_4$ receptor -mediated responses in various tissues. In addition, the coupling of each 5-HT$_4$ receptor isoform to its effector may be influenced by the cellular environment. In support of this hypothesis, we have recently shown that functional potency of h5-HT$_{4(e)}$ receptor ligands was dependent on the cellular context in which the receptor was expressed (Mialet *et al.*, 2000). It is important therefore to analyse the effects of different compounds at individual 5-HT$_4$ receptor subtypes measuring the same second messenger response in cells of the same genetic background. This need is further highlighted by the recent increased interest in the therapeutic utility of selective 5-HT$_4$ ligands to treat a variety of disorders such as cardiac arrythmias (Kaumann, 1994, Rahme *et al.*, 1999), neurodegenerative diseases (Reynolds *et al.*, 1995; Wong *et al.*, 1996), irritable bowel syndrome (Hedge & Eglen, 1996), and urinary incontinence (Boyd & Rohan, 1994).

To date there has been no detailed and comprehensive pharmacological study to characterise and compare the effects of different 5-HT$_4$ ligands on different h5-HT$_4$ receptor isoforms. The present study was therefore undertaken to determine the pharmacological profile of the h5-HT$_{4(d)}$ receptor and compare the potencies and intrinsic activities of 5-HT$_4$ serotoninergic agents using the elevation of cAMP as a functional measure of compound activity. The h5-HT$_{4(d)}$ receptor subtype is of particular interest for several reasons. Firstly, in contrast to h5-HT$_{4(a)}$, h5-HT$_{4(b)}$ and h5-HT$_{4(e)}$ receptors which are human counterparts of rat (Gerald *et al.*, 1995; Claeysen *et al.*, 1999) and mouse (Claeysen *et al.*, 1996; Claeysen *et al.*, 1999) 5-HT$_4$ receptors, the h5-HT$_{4(d)}$ receptor isoform has not been yet described in any other species. Secondly, expression of the h5-HT$_{4(d)}$ receptor was only restricted to the gut (Blondel *et al.*, 1998) and therefore it could serve as a drug target for the treatment of gastrointestinal associated disorders such as the irritable bowel syndrome. Finally, the h5-HT$_{4(d)}$ receptor isoform corresponds to an ultrashort form of the receptor with a truncation of the carboxyl terminus with only two amino acids after the splicing site (Blondel *et al.*, 1998).

In this paper, we stably expressed the h5-HT$_{4(d)}$ receptor in Chinese hamster ovary (CHO) cells and showed that it displayed a typical 5-HT$_4$ binding profile with the radiolabelled specific antagonist [^{3}H]-GR113808 and a series of 5-HT$_4$ displacing ligands. However, by comparison with our previous functional study in CHO cells stably expressing the h5-HT$_{4(e)}$ receptor (Mialet *et al.*, 2000), we report striking differences in its potency in stimulating cAMP formation in response to various standard 5-HT$_4$ ligands.

Materials and Methods

Cell culture

Cell culture materials and reagents were obtained from Life Technologies (Cergy Pontoise, France). CHO cell line was purchased from ATCC (Rockville, USA) and was grown at 37°C and 5% CO_2 in HamsF12 medium supplemented with 10% foetal calf serum, 10 mM HEPES (pH 7.4) and antibiotics.

Stable expression of the h5-HT$_{4(d)}$ receptor in CHO cells

The full coding region of h5-HT$_{4(d)}$ receptor was subcloned into the expression vector pRC/CMV containing the neomycin selection gene (Invitrogen, Carlsbad, CA, USA). Briefly, confluent cells were transfected with 10 μg of the expression vector by electroporation using a gene pulser transfection apparatus (Biorad, Ivry sur Seine, France; setting 960 μF, 250 V). Forty eight hours after the transfection period neomycin (1.25mg ml^{-1}) was added to the dishes for selection. The antibiotic-containing medium was replaced every 2-3 days over 2 weeks. Isolated colonies were selectively trypsinized for further selection, subcloning and propagation of cell clones. h5-HT$_{4(d)}$ receptor expressing clones were detected both by their ability to stimulate cAMP production after treatment with 5-HT and to bind a specific 5-HT$_4$ antagonist, [^{3}H]GR 113808.

Cyclic AMP radioimmunoassay

For measurement of intracellular cAMP production, stably transfected CHO cells grown to confluence in 24-well plates were incubated overnight with Ham-F12 medium containing 5% dialysed-FCS. At the beginning of the assay, CHO cells were preincubated for 15 min in serum-free medium supplemented with 5 mM theophylline, 10 μM pargyline and 1 μM GR127935 to block the activity of endogenous 5-HT$_{1B}$ receptors. 5-HT$_4$ antagonists were also

incubated during this preincubation period. 5-HT or other serotoninergic agonists were then added for an additional 15 min. The reaction was stopped by aspiration of the medium and addition of 50 µl ice-cold perchloric acid (20%). After a 30 min period, neutralisation buffer was added (HEPES 25mM, KOH 2N) and supernatant was extracted after centrifugation at 10,000 rpm for 5 min. cAMP was quantified using a radioimmunoassay kit (cAMP competitive radioimmunoassay, Immunotech, Marseille, France). Student's *t*-tests were performed using the QuickTTest software.

Membrane preparation and radioligand binding assays

Membrane preparation and radioligand binding assays were performed as previously described (Blondel *et al.*, 1998, Mialet *et al.*, 2000). Briefly, cells grown at confluence were washed twice with Phosphate-Buffered Saline (PBS) and centrifuged at 300 g for 5 min. The resulting pellet was resuspended in 1 ml of ice-cold HEPES buffer (50 mM, pH 7.4), centrifuged at 40,000 g for 15 min at 4°C. The final pellet was resuspended in 1 ml HEPES buffer and protein concentrations were determined by the method of Bradford (1976).

Radioligand binding assays were performed in 500 µl buffer (50 mM HEPES, pH 7.4) containing 20 µl of [^{3}H]-GR113808, 50 µg of membrane preparation and 20 µl of displacing drug. Saturation experiments were performed using [^{3}H]-GR113808 at nine concentrations ranging from 0.01 to 4 nM. Non specific binding was measured in the presence of 10 µM ML10375 and subtracted from total binding to determine the affinity of [^{3}H]-GR113808 for its receptor (K_d, nM) and the total number of receptors (B_{max}, fmol mg^{-1} protein). Competition assays were performed in the presence of nine concentrations of the displacing ligands (10^{-12} to 10^{-4} M) and a concentration of 0.2 nM of [^{3}H]-GR113808. Incubations were performed at 25°C for 30 min and the reaction was terminated by rapid filtration through Whatman GF/B filter paper using the Brandel model 48R cell harvester. Radioactivity was measured using a

Beckman model LS 6500C liquid scintillation counter. Binding data were analysed by computer-assisted nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA).

Drugs

GR113808 ([1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate) and GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'- methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide) were gifts from Glaxo Research Group (Ware, Hertfordshire, UK). [^{3}H]-GR113808 was purchased from Amersham (Orsay, France). ML10302 (2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride) and ML10375 (2-[*cis*-3,5-dimethylpiperidino]ethyl 4-amino-5-chloro-2-methoxybenzoate) were synthesised as previously described (Langlois *et al.*, 1994; Yang *et al.*, 1997). 5-HT (5-hydroxytryptamine) and 5-MeOT (5-methoxytryptamine) were from Aldrich (L'Isle d'Abeau Chesnes, France). BIMU1 (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide) and cisapride (*cis*-4-amino-5-chloro-*N*-[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide) were synthesised in our laboratory. Renzapride (BRL 24924) ((±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride) and SB204070 (8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride) were generously given by SmithKline Beecham (Harlow, U.K.). RS23597 (3-(piperidine-1-yl)-propyl-4-amino-5-chloro-2-methoxybenzoate hydrochloride), RS39604 (1-(5-chloro-2(3,5-dimethoxy)benzyloxy-4-aminophenyl)-3-(N-(methylsulfamido)ethyl-4-piperidyl)propanone) and RS67333 (1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-n-butyl-4-piperidinyl1-propanone) were from Tocris Interchim (Montluçon, France).

Results

Binding studies of the h5-HT$_{4(d)}$ receptor

The cDNA encoding the h5-HT$_{4(d)}$ receptor was stably expressed in CHO cells at 493±25 fmol mg^{-1} protein. Saturation analysis revealed a single saturable binding site with high affinity for [^{3}H]-GR113808 (Figure 1). Non-specifc binding increased linearly with increasing ligand concentration (Figure 1). The K_d value for the transfected h5-HT$_{4(d)}$ receptor was 0.30±0.05 nM, respectively (Figure 1, Table 1) and correlated with those obtained in COS-7 cells transiently transfected with the different h5-HT$_4$ receptors (Blondel *et al.*, 1998). It was also close to those found in CHO and C6-glial cells stably expressing the h5-HT$_{4(e)}$ receptor (Mialet *et al.*, 2000).

(Figure 1 and Table 1 near here)

Since the amino sequences of 5-HT$_4$ receptor splice variants are identical in the transmembrane regions (Blondel *et al.*, 1998; Gerald *et al.*, 1995), it was anticipated that there would be no major pharmacological differences at the level of binding between the h5-HT$_{4(d)}$ receptor isoform and the h5-HT$_{4(e)}$ receptor previously studied in the same cellular system (Mialet *et al.*, 2000). This was verified with a number of standard 5-HT$_4$ receptor ligands (Table 1). A range of 5-HT$_4$ receptor agonists and antagonists completely inhibited the specific binding of [^{3}H]-GR113808 in CHO membranes expressing the h5-HT$_{4(d)}$ receptor (Figure 2). All the displacement curves were monophasic, giving a Hill coefficient not different from 1. The data summarised in Table 1 demonstrate that the pharmacological profile of the h5-HT$_{4(d)}$ receptor in terms of rank order of affinity of the different ligands

tested is similar to those found for native 5-HT$_4$ receptors as studied *in vivo* in human atria (Kaumann *et al.*, 1996), rat striatum (Langlois *et al.*, 1994; Yang *et al.*, 1997), mouse colliculi (Ansanay *et al.*, 1996), or after expression of cloned human, mouse or rat 5-HT$_4$ receptor isoforms in COS-7 (Gerald *et al.*, 1995; Blondel *et al.*, 1997; 1998; Claeysen *et al.*, 1996; 1999) and CHO cell lines (Mialet *et al.*, 2000). The rank order of apparent agonist and antagonist affinities were, respectively, RS67333> ML10302> BIMU1> 5-HT = cisapride> renzapride> 5-MeOT for the h5-HT$_{4(d)}$ receptor (Figure 2, Table 1). However, we found that BIMU1 had a lower affinity (about 2 fold) for the h5-HT$_{4(d)}$ receptor than the h5-HT$_{4(e)}$ receptor (Ki = 64 nM) stably transfected in CHO cells (Table 1, Mialet *et al.*, 2000).

(Figure 2 near here)

Functional coupling of the h5-HT$_{4(d)}$ receptor isoform

Functional coupling of the recombinant h5-HT$_{4(d)}$ receptor isoforms to adenylyl cyclase was investigated by measuring cAMP production in response to various 5-HT$_4$ receptor ligands (Figure 3). All 5-HT$_4$ agonists used in these experiments stimulated cAMP production in a dose dependent manner in expressing the 5-HT$_{4(d)}$ receptor isoform (Figure 3A). However, we were not able to rank EC_{50} values of 5-HT$_4$ agonists obtained from adenylyl cyclase functional assays in CHO cells. Indeed, EC_{50} values of ML10302, BIMU1, cisapride, 5-HT and renzapride were similar (Table 2). Furthermore, they were lower than those previously obtained with the 5-HT$_{4(e)}$ receptor indicating that the 5-HT$_{4(d)}$ receptor was more efficiently coupled to its effector than the 5-HT$_{4(e)}$ receptor isoform (Table 2, Mialet *et al.*, 2000). At the 5-HT$_{4(d)}$ receptor, 5-MeOT was the most potent ligand to induce cAMP production since its EC_{50} value was 1.1±0.2 nM. With regard to agonist efficiencies, renzapride was the only agonist found to display full agonist properties at the 5-HT$_{4(d)}$ receptor (Figure 3A, Table 2).

5-HT$_4$ antagonists were tested for their ability to antagonise 5-HT-induced cAMP production (Figure 3B; Table2). GR113808 (3 nM) and ML10375 (10 nM) produced a rightward shift in 5-HT dose response curve (Figure 3B) and were approximately equipotent (Table 2). Surprisingly, we were unable to generate a dose-response curve for 5-HT in the presence of the two highly specific 5-HT$_4$ antagonists, SB204070 and RS39604 (see below).

(Figure 3 and Table 2 near here)

Constitutive activity of the h5-HT$_{4(d)}$ receptor

A constitutive activity of the h5-HT$_{4(d)}$ receptor was observed in the absence of any 5-HT$_4$ ligand (Figure 4). Indeed, the absolute value for cAMP production in untransfected CHO cells and under basal conditions was 4.3±0.8 pmoles cAMP/well (Figure 4). In CHO cells stably expressing the h5-HT$_{4(d)}$ receptor isoform, cAMP content reached 17.5±2.8 pmoles/well, (Figure 4). This effect was not due to the presence of 5-HT in culture medium since cells were cultured overnight before the assay with dialysed FCS-containing medium. These data clearly indicate that expression of the h5-HT$_{4(d)}$ receptor induced a spontaneously active receptor state.

Since constitutive activation of G-protein-coupled receptors is often related to inverse agonist properties, we tested the effects of potent selective 5-HT$_4$ receptors antagonists on the intrinsic activity of the h5-HT$_{4(d)}$ receptor (Figure 4). ML10375 (1 μM) and GR113808 (1 μM) which behaved as inverse agonists at the h5-HT$_{4(e)}$ receptor (Mialet *et al*., 2000) were neutral antagonists and had no effect on the constitutive activity of the h5-HT$_{4(d)}$ receptor (Figure 4). These experiments show that the observed inverse agonist effect of GR113808 and ML10375 is dependent on the considered isoform.

In addition, RS39604 and SB204070 usually described as potent 5-HT$_4$ antagonists in various biological models such as mouse colliculi neurones (Claeysen *et al*., 1998) acted as partial agonists for the recombinant h5-HT$_{4(d)}$ receptor (Figure 4). Indeed, concentration response curves clearly showed that SB204070 (Figure 5A) and RS39604 (Figure 5B) were able to stimulate cAMP production at the h5-HT$_{4(d)}$ receptor. These effects resulted from 5-HT$_4$ receptor activation because they were antagonised by the selective neutral 5-HT$_4$ antagonist ML10375 (1 μM) (Figure 5). However, all the 5-HT$_4$ antagonists tested in the study, significantly inhibited 5-HT-induced cAMP production at the h5-HT$_{4(d)}$ receptor (Figure 4).

(Figure 4 and Figure 5 near here)

Discussion

h5-HT$_4$ receptor isoforms are generated by splicing events occurring both in the second extracellular loop and the C terminus of the h5-HT$_4$ receptor just after the amino acid Leu^{358} (Blondel *et al*., 1997; 1998; Bender *et al*., 2000; Claeysen *et al*., 1997; 1999; Mialet *et al*., 2000; Van den Wyngaert *et al*., 1997). Unfortunately it is not possible to study individual h5-HT$_4$ receptor in native tissues due to the lack of complete segregation of the receptors in the central nervous system and the periphery (Blondel *et al*., 1998; Mialet *et al*., 2000). The use of recombinant expression systems is therefore a useful approach and also allows the study of human receptors. To analyse the influence of the C-terminal tail on pharmacological properties of the h5-HT$_{4(d)}$ receptor, binding studies and the functional profile of this recombinant h5-HT$_4$ receptor isoform stably expressed in the CHO cell line was determined using a number of standard 5-HT$_4$ agonists and antagonists.

Saturation binding assays with the specific radiolabelled antagonist [^{3}H]-GR113808 showed a K_d value for the h5-HT$_{4(d)}$ receptor (Figure 1) which is in good agreement with those reported in human atria (Kaumann *et al*., 1996), in human brain membranes (Waeber *et al*., 1993), and in COS-7 and CHO cell lines transfected with other h5-HT$_4$ receptor splice variants (Bender *et al*., 2000; Blondel *et al*., 1998; Claeysen *et al*., 1997, Mialet *et al*., 2000, Van den Wyngaert *et al*., 1997). The pharmacological binding profile determined in competition studies revealed a rank order of affinity of 5-HT$_4$ compounds for the h5-HT$_{4(d)}$ receptor (Figure 2, Table 1) that was consistent with those previously reported in various biological models such as rat striatum and mouse colliculi (Ansanay *et al*., 1996, Yang *et al*., 1997). In addition, it matched that obtained in CHO cells expressing the h5-HT$_{4(e)}$ receptor (Mialet *et al*., 2000). The ligand found to have the highest binding affinity for the h5-HT$_{4(d)}$ receptor was the benzodioxane derivative, SB204070 (Table 1). On the other hand, 5-HT and 5-MeOT showed the lowest affinity (Table 1). These results correlate well with those

obtained in binding experiments with the h5-HT$_{4(e)}$ receptor expressed in CHO and C6-glial cells (Mialet *et al*., 2000) and suggest that C-terminal tails of h5-HT$_4$ receptor splice variants do not influence binding properties of 5-HT$_4$ ligands.

Interestingly, we found that the rank order of potencies of 5-HT$_4$ agonists did not parallel their rank order of binding affinities. Recently, we also noted significantly lower agonist potencies relative to their binding affinities for the h5-HT$_{4(e)}$ receptor stably expressed in CHO and C6-glial cells (Table 2 from Mialet *et al.*, 2000). This is not specific for the h5-HT$_4$ receptor since similar observations have already been reported for rat 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptors, and human 5-HT$_7$ receptors when expressed in different cell lines (Adam *et al*., 1998; Gerald *et al*., 1995). A possible explanation for the discrepancy between agonist functional potency and binding affinity is that [^{3}H]-GR113808 labels a different agonist affinity state of the receptor compared to that mediating the functional response. Alternatively, it has been speculated that some ligands might differentially activate a receptor reserve (Gerald *et al*., 1995).

In contrast to binding studies, very striking differences in functional studies were observed between the pharmacological profile of the h5-HT$_{4(d)}$ receptor and that previously determined for the h5-HT$_{4(e)}$ receptor in the same cellular system (Table 2, Mialet *et al*., 2000). Overall, the h5-HT$_{4(d)}$ receptor displayed equipotent coupling for all the agonists tested in this study (EC_{50} in the range of 1 to 6 nM, Figure 3, Table 2). On the other hand, the h5-HT$_{4(e)}$ receptor isoform was less potent than the h5-HT$_{4(d)}$ receptor to increase cAMP accumulation in response to 5-HT$_4$ agonists (EC_{50} in the range of 7 to 180 nM, Table 2, Mialet *et al*., 2000). For instance, renzapride was almost twenty times more potent at the h5-HT$_{4(d)}$ receptor than we previously observed at the h5-HT$_{4(e)}$ receptor (Table 2). These results appear not to be dependent on receptor expression since according to a previous study from our laboratory the

h5-HT$_{4(e)}$ receptor was expressed in CHO cells at a density which was close to that of the h5-HT$_{4(d)}$ receptor found in this study (Mialet *et al*., 2000).

Our data show that structural differences in the C-terminal tails of h5-HT$_4$ receptors influence and contribute to the specificity of their functional pharmacological profile. The second and third intracellular loops of G protein coupled receptors (GPCRs) are defined as being the regions that physically interact with G proteins and are required for effective coupling. This has been shown for various GPCRs such as the α_2-adrenergic receptor (Eason & Ligget, 1996) and the 5-HT$_7$ receptor (Obosi *et al.*, 1997). But recent data indicate that C-terminal sequences of GPCRs also contribute to the potency of coupling between the receptor and its effector, as reported for the dopamine D1 (Jensen *et al.*, 1996; Sugamori et al., 1998) and the angiotensin II receptors (Sano *et al.*, 1997). The fact that the h5-HT$_{4(e)}$ receptor is less coupled to Gs proteins than the h5-HT$_{4(d)}$ receptor also suggest that these splice variants could be coupled either to distinct isoforms of Gs proteins with different coupling efficiencies to adenylyl cyclase or to different signal transduction cascades. In support of this hypothesis, Namba and colleagues (1993) have shown that the isoforms of the prostaglandin EP3 receptor, which differ only at their C-terminal tails and are produced by alternative splicing, couple to different G proteins to activate different messenger systems without changing ligand binding specificity. The possibility that h5-HT$_4$ receptor splice variants might regulate distinct signalling pathways is currently under investigation.

Another interesting observation from this study is that RS39604 and SB204070 behaved as partial agonists in CHO cells expressing the h5-HT$_{4(d)}$ receptor although they were described as highly potent antagonists in various biological models like guinea-pig distal colon (Gaster & Sanger, 1994; Wardle *et al.*, 1994), rat oesophagus (Hegde *et al.*, 1995), and mouse colliculi neurones (Claeysen *et al.*, 1998). Our results are in accordance with those of

Claeysen and colleagues (1998) who found partial agonistic activities of these two 5-HT$_4$ compounds in COS-7 cells expressing the h5-HT$_{4(a)}$ receptor.

In fact, to explain the discrepancies between *in vitro* and *in vivo* data, one could speculate that several 5-HT$_4$ receptor isoforms expressed in a given tissue could form heterodimers with distinct functional pharmacological properties. This molecular mechanism has been recently described for other GPCR such as the opioid receptors (Jordan & Devi, 1999). Yet, tissue specific proteins could interact with the intracellular C-terminal tails of h5-HT$_4$ receptors and influence their intracellular signalling. It is interesting to note that the new cloned h5-HT$_{4(e)}$ receptor splice variant displays in its C-terminal end a consensus motif for PDZ-domain containing proteins (Claeysen *et al*., 1999; Mialet *et al*., 2000). These proteins with PDZ-domains act as molecular organizers that cluster receptors in the plasma membrane or mediate direct interaction with GPCR to influence their intracellular events (Saras & Heldin, 1996). Whether such mechanisms occur for h5-HT$_4$ receptor splice variants and contribute to the specificity of their pharmacological profile in a given tissue has yet to be elucidated.

Finally, we found that the h5-HT$_{4(d)}$ receptors expressed in CHO cells were constitutively activated when expressed at physiological densities (Figure 4). Such constitutive coupling has already been reported for the h5-HT$_{4(e)}$ receptor stably expressed in CHO cell line (Mialet *et al*., 2000) and for recombinant mouse, rat and h5-HT$_{4(e)}$ receptors transfected in COS-7 cells (Claeysen *et al*., 1999). It has been shown that the constitutive activity of mouse 5-HT$_4$ short splice variants is higher than that of the long C-terminal sequence variants (Claeysen *et al*., 1999). However, we found that the h5-HT$_{4(d)}$ receptor which has the shortest C-terminal tail displayed a similar constitutive activity (Figure 4) than the h5-HT$_{4(e)}$ receptor (Mialet *et al*., 2000). Therefore in our hands, we cannot conclude about the influence of the length of the C-terminal tail on constitutive activation.

Some interesting features were found with inverse agonist properties of several 5-HT$_4$ ligands (Figure 4). The highly potent 5-HT$_4$ antagonists, GR113808 and ML10375, were neutral antagonist at the 5-HT$_{4(d)}$ receptor but on the contrary acted as inverse agonists at the 5-HT$_{4(e)}$ receptor (Table 1, Mialet *et al*., 2000). These results clearly indicate that the C-terminal tails of the 5-HT$_4$ receptor isoforms may strongly influence the inverse agonist properties of a given 5-HT$_4$ ligand.

In conclusion, we have determined the pharmacological profile of the h5-HT$_{4(d)}$ receptor stably expressed in CHO cells. We did not observe any major difference in the binding properties of 5-HT$_4$ ligands between the h5-HT$_{4(d)}$ receptor and other h5-HT$_4$ receptor isoforms. However, striking differences were observed in functional studies and we have presented evidence that C-terminal tails of 5-HT$_4$ receptor isoforms may directly influence their functional properties. Further detailed pharmacological characterisations of other cloned h5-HT$_4$ receptor splice variants in heterologous expression systems will facilitate the interpretation of *in vivo* responses to 5-HT$_4$ ligands in native tissues.

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References

ADHAM, N., ZGOMBICK, J. M., BARD, J. & BRANCHEK, T. A. (1998) Functional characterisation of the recombinant human 5-hydroxytryptamine7(a) receptor isoform coupled to adenylate cyclase stimulation. *J. Pharmacol. Exp. Ther.*, **287**, 508-514.

ANSANAY, H., SEBBEN, M., BOCKAERT, J. & DUMUIS A. (1996) Pharmacological comparison between [^{3}H]-GR113808 binding sites and functional 5-HT$_4$ receptors in neurons. *Eur. J. Pharmacol.*, **298**, 165-174.

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

BENDER, E., PINDON, A., VAN OERS, I., ZHANG, Y. B., GOMMEREN, W., VERHASSELT, P., JURZAK, M., LEYSEN, J. & LUYTEN, W. (2000) Structure of the human serotonin 5-HT$_4$ receptor gene and cloning of a novel 5-HT$_4$ splice variant. *J. Neurochem.*, **74**, 478-489.

BLONDEL, O., GASTINEAU, M., DAHMOUNE, Y., LANGLOIS, M. & FISCHMEISTER, R. (1998) Cloning, expression and pharmacology of four human 5-HT$_4$ receptor isoforms produced by alternative splicing in the carboxyl terminus. *J. Neurochem.*, **70**, 2252-2261.

BLONDEL, O., VANDECASTEELE, G., GASTINEAU, M., LECLERC, S., DAHMOUNE, Y., LANGLOIS, M. & FISCHMEISTER, R. (1997) Molecular and functional characterisation of a 5-HT$_4$ receptor cloned from human atrium. *FEBS Lett.*, **412**, 465-474.

BOCKAERT, J., SEBBEN, M. & DUMUIS, A. (1990) Pharmacological characterisation of 5-hydroxytryptamine4(5-HT4) receptors positively coupled to adenylate cyclase in adult guinea pig hippocampal membranes: effect of substituted benzamide derivatives. *Mol. Pharmacol.*, **37**, 408-411.

BOYD, I. W. & ROHAN, A. P. (1994) Urinary disorders associated with cisapride. *Med. J. Aust.* **160**, 579-580.

BRADFORD, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.

CLAEYSEN, S., FAYE, P., SEBBEN, M., LEMAIRE, S., BOCKAERT, J. & DUMUIS, A. (1997) Cloning and expression of human 5-HT$_{4S}$ receptors. Effect of receptor density on their coupling to adenylyl cyclase. *Neuroreport*, **8**, 3189-3196.

CLAEYSEN, S., FAYE, P., SEBBEN, M., TAVIAUX, S., BOCKAERT J. & DUMUIS, A. (1998) 5-HT$_4$ receptors: cloning and expression of new splice variants. *Ann. N. Y. Acad Sci.*, **861**, 49-56.

CLAEYSEN, S., SEBBEN, M., BECAMEL, C., BOCKAERT, J. & DUMUIS, A. (1999) Novel brain-specific 5-HT$_4$ receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. *Mol Pharmacol.*, **55**, 910-920.

CLAEYSEN, S., SEBBEN, M., JOURNOT, L., BOCKAERT, J. & DUMUIS, A. (1996) Cloning, expression and pharmacology of the mouse 5-HT$_{4L}$ receptor. *FEBS Lett.,* **398**, 19-25.

DUMUIS, A., BOUHELAL, R., SEBBEN, M., CORY, R. & BOCKAERT, J. (1988) A non classical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous systeme. *Mol. Pharmacol.*, **34**, 880-887.

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 neurons. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 403-410.

EASON, M. G. & LIGGET, S. B. (1996) Chimeric mutagenesis of putative G-protein coupling domains of the α_{2A}-adrenergic receptor. *J. Biol. Chem.,* **271,** 12826-12832.

EGLEN, R. M., WONG, E. H. F., DUMUIS, A. & BOCKAERT, J. (1995) Central 5-HT$_4$ receptors. *Trends Pharmacol. Sci.*, **16**, 391-397.

GASTER, L.M. & SANGER, G.J. (1994) SB204070: 5-HT receptor antagonists and their potential therapeutic utility. *Drugs Future*, **19**, 1109-1121.

GERALD, C., ADHAM, N., KAO, H. T., OLSEN, M. A., LAZ, T. M., SCHECHTER, L. E, BARD, J. E., VAYSSE, P. J. J., HARTIG, P. R., BRANCHEK, T. A. & WEINSHANK R. L. (1995) The 5-HT$_4$ receptor: molecular cloning and pharmacological characterisation of two splice variants. *EMBO J.*, **14**, 2806-2815.

GERHARDT, C. C. & VAN HEERIKHUIZEN, H. (1997) Functional characteristics of heterologously expressed 5-HT receptors. *Eur. J. Pharmacol.,* **334,** 1-23.

HEGDE, S., BONHAUS, D.W., JOHNSON, L.G., LEUNG, E., CLARK, R.D. & EGLEN, R.M. (1995) RS39604: a potent, selective and orally active 5-HT$_4$ receptor antagonist. *Br. J. Pharmacol.*, **115,** 1087-1095.

HEGDE, S. & EGLEN, R. (1996) Peripheral 5-HT$_4$ receptors. *FASEB J.*, **10**, 1398-1407.

JENSEN, A. A., PEDERSEN, U. B., DIN, N. & ANDERSEN, P. H. (1996) The dopamine D1 receptor family: structural and functional aspects. *Biochem. Soc. Trans.,* **24,** 163-169.

JORDAN, B. A. & DEVI, L. A. (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature,* **399**, 697-700.

KAUMANN, A. J. (1994) Do human atrial 5-HT$_4$ receptors mediate arrhythmias? *Trends Pharmacol. Sci.*, **15**, 451-455.

KAUMANN, A. J., LYNHAM, J. A. & BROWN A. M. (1996) Comparison of the densities of 5-HT$_4$ receptors, β1- and β2-adrenoceptors in human atrium: functional implications. *Naunyn-Schmied. Arch. Pharmacol.,* **353**, 592-595.

LANGLOIS, M., ZHANG, L., YANG, D., BRÉMONT, B., SHEN, S., MANARA, L. & CROCI T. (1994). Design of a potent 5-HT$_4$ receptor agonist with nanomolar affinity. *Biomed. Chem. Lett.* **4**, 1433-1436.

MIALET, J., BERQUE-BESTEL, I., EFTEKHARI, P., GASTINEAU, M., GINER, M., DAHMOUNE, Y., DONZEAU-GOUGE, P., HOEBEKE, J., LANGLOIS, M., SICSIC, S., FISHMEISTER, R. & LEZOUALC'H, F. (2000) Isolation of the serotoninergic 5-HT$_{4(e)}$ receptor from human heart and comparative analysis of its pharmacological profile in C6-glial and CHO cells. *Br. J. Pharmacol.*, **129**, 771-781.

NAMBA, T., SUGIMOTO,Y., NEGISHI, M., IRIE, A., USHIKUBI, F., KAKIZUKA, A., ITO, S., ICHIKAWA, A. & NARUMIYA, S. (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature*, **365**, 166-170.

OBOSI, L. A., HEN, R., BEADLE, D. J., BERMUDEZ, I. & KING, L. A. (1997) Mutational analysis of the mouse 5-HT7 receptor: importance of the third intracellular loop for receptor-G-protein interaction. *FEBS Lett.*, **412**, 321-324.

OUADID, H., SEGUIN, J., DUMUIS, A., BOCKAERT, J. & NARGEOT, J. (1991) Serotonin increases calcium current in human atrial myocytes via the newly described 5-hydroxytryptamine 4 receptors. *Mol. Pharmacol.*, **41**, 346-351.

RAHME, M. M., COTTER, B., LEISTAD, E., WADHWA, M. K., MOHABIR, R., FORD, A. P. D. W., EGLEN, R. M. & FELD, G. K. (1999) Electrophysiological and antiarrhythmic effects of the atrial selective antagonist RS-100302 in experimental atrial flutter and fibrillation. *Circulation*, **100**, 2010-2017.

REYNOLDS, G. P., MASON, L., MELDRUM, A., DE KECZER, S., PARNES, H. & WONG E. H. F. (1995) 5-hydroxytryptamine (5-HT)$_4$ receptors in post mortem human brain tissue: distribution, pharmacology and effects of neurodegenerative diseases. *Br. J. Pharmacol.*, **114**, 993-998.

SANO, T., OHYAMA, K., YAMANO, Y., NAKAGOMI, Y., NAKAZAWA, S., KIKYO, M., SHIRAI, H., BLANK, J. S., EXTON, J. H. & INAGAMI, T. (1997) A domain for G protein coupling in carboxyl-terminal tail of rat angiotensin II receptor type 1A. *J. Biol. Chem.*, **272**, 23631-23636.

SARAS, J. & HELDIN, C. H. (1996) PDZ domains bind carboxy-terminal sequences of target proteins. *Trends Biochem. Sci.*, **21**, 455-458.

SAXENA, P. R. (1995) Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacol. Ther.*, **66**, 339-368.

SUGAMORI, K. S., SCHEIDELER, M. A., VERNIER, P. & NIZNIK, H. B. (1998) Dopamine D_{1B} receptor chimeras reveal modulation of partial agonist activity by carboxy-terminal tail sequences. *J. Neurochem.*, **71**, 2593-2599.

VAN DEN WYNGAERT, I., GOMMEREN, W., VERHASSELT, P., JURZAK, M., LEYSEN, J., LUYTEN, W. C. & BENDER, E. (1997) Cloning and expression of a human serotonin 5-HT$_4$ receptor cDNA. *J. Neurochem.*, **69**, 1810-1819.

WAEBER, C., SEBBEN, M., GROSSMAN, C., JAVOY-AGID, F., BOCKAERT, J. & DUMUIS A. (1993) [3H]-GR113808 labels 5-HT$_4$ receptors in the human and guinea-pig brain. *Neuroreport*, **4**, 1239-1242.

WARDLE, K. A., ELLIS, E.S., BAXTER, G.S., KENNETT, G.A., GASTER, L.M. & SANGER, G.J. (1994) The effects of SB204070, a highly potent and selective 5-HT4 receptor antagonist, on guinea-pig distal colon. *Br. J. Pharmacol.*, **112**, 789-794.

WARDLE, K. A. & SANGER, G. J. (1993) The guinea-pig distal colon: a sensitive preparation for the investigation of 5-HT$_4$ receptor-mediated contractions. *Br. J. Pharmacol.*, **110**, 1593-1599.

WONG, E. H., REYNOLDS, G. P., BONHAUS, D. W., HSU, S. & EGLEN R. M. (1996) Characterisation of [^{3}H]-GR113808 binding to 5-HT$_4$ receptors in brain tissues from patients with neurodegenerative disorders. *Behav. Brain Res.*, **73**, 249-252.

YANG, D., SOULIER, J.L., SICSIC, S., MATHÉ-ALLAINMAT, M., BRÉMONT, B., CROCI, T., CARDAMONE, R., AUREGGI, G. & LANGLOIS M. (1997) New esters of 4-amino-5-chloro-2-methoxybenzoic acid as potent agonists and antagonists for 5-HT$_4$ receptors. *J. Med. Chem.* **40**, 608-621.

Table 1. Affinities of various 5-HT$_4$ ligands that compete with the binding of 0.2 nM [^{3}H]GR113808 in CHO cells stably expressing the h5-HT$_{4(d)}$ receptor

	h5-HT$_{4(d)}$
	K_i (nM) (±S.E.M.)
AGONISTS	
5-HT	305±58
5-MeOT	1365±308
ML10302	12±3
Renzapride	583±92
Cisapride	359±91
RS67333	6.5±1.5
BIMU1	64±16
ANTAGONISTS	
GR113808	0.12±0.03
ML10375	0.92±0.21
RS23597	67±14
RS39604	6.8±1.3
SB204070	0.16±0.03

Ki values were obtained from competition binding assays with 0.2 nM [^{3}H]GR113808 in CHO cells stably transfected with the h5-HT$_{4(d)}$ receptor. IC_{50} values correspond to a displacement of 50% of specific radioligand binding and were converted to K_i values according to the equation: $K_i = IC_{50}/(1+(\text{concentration of } [^3H]GR113808/K_d))$. The data were obtained using nine concentrations of ligand and all experiments were performed in triplicate.

Table 2. Effects of 5-HT$_4$ ligands on cAMP production in CHO cells expressing the h5-HT$_{4(d)}$ receptor

	h5-HT$_{4(d)}$		**h5-HT$_{4(e)}$** (from Mialet *et al*., 2000)	
AGONISTS	**EC$_{50}$** (nM)	**E$_{max}$** (% of 5-HT maximal response)	**EC$_{50}$** (nM)	**E$_{max}$** (% of 5-HT maximal response)
5-HT	5.3±0.6	100	25±6	100
5-MeOT	1.1±0.2	64±2	7.0±0.7	108±2
Renzapride	5.8±1.1	105±5	107±41	66±6
Cisapride	5.2 ±1.6	87±4	180±35	85±4
ML10302	4.4±1.3	39±3	51±23	30±3
BIMU1	5.9±1.4	73±4	52±23	47±5
ANTAGONISTS	**EC$_{50}$** (nM)	**K$_b$** (nM)	**EC$_{50}$** (nM)	**K$_b$** (nM)
GR113808	175±34 (3 nM)	0.094	79±12 (3 nM)	1.38
ML10375	52±15 (10 nM)	1.1	74±17 (1 nM)	1.01

cAMP production was measured in CHO cells expressing the h5-HT$_{4(d)}$ receptor. EC$_{50}$ values correspond to the concentration of agonist required to obtain half-maximal stimulation of adenylyl cyclase. The maximum response produced by each drug was normalised to the 5-HT induced maximum response. For the antagonists, EC$_{50}$ values correspond to the concentration of 5-HT required to produce half-maximal stimulation of adenylyl cyclase in the presence of the indicated concentration of antagonist. K$_b$ values were calculated with the formula: K$_b$=[B]/[(A'/A)-1] where [B] is the concentration of antagonist and A' and A the EC$_{50}$ values of 5-HT measured respectively in the presence and absence of antagonist. Values are means ± S.E.M. of three experiments performed in triplicate. They were analysed by non-linear regression analysis. Data for the h5-HT$_{4(e)}$ receptor are from Mialet *et al*. (2000).

Figure Legends

Figure 1. Saturation analysis of [^{3}H]-GR113808 binding to the h5-HT$_{4(d)}$ receptor stably expressed in CHO cells.

Membranes harvested from stably transfected CHO cells expressing the h5-HT$_{4(d)}$ receptor were incubated with nine concentrations of [^{3}H]-GR113808 for 30 min at 25°C. Non-specific binding was determined with 10 μM ML10375. Results are from single experiments but are representative of three such experiments.

Figure 2. Inhibition of specific [^{3}H]-GR113808 binding to the h5-HT$_{4(d)}$ receptor expressed in CHO cells.

Membranes from CHO cells stably expressing the h5-HT$_{4(d)}$ (A, B) receptor were incubated with 0.2 nM [^{3}H]-GR113808 in the presence of increasing concentrations of 5-HT$_4$ agonists (A) or antagonists (B). Non specific binding was defined by 10 μM ML10375. Data are presented as a percentage of specific binding in the absence of displacing drug. Results are from single experiments but are representative of three such experiments using a range of nine concentrations of ligands. Data were analysed by computer-assisted non-linear regression analysis (GraphPad, Prism Software). The corresponding K_i values are presented in Table 1.

Figure 3. Stimulation of cAMP accumulation in CHO cells stably expressing the h5-HT$_{4(d)}$ receptor by various 5-HT$_4$ ligands.

cAMP measurements were performed on the h5-HT$_{4(d)}$ receptor (A, B) as described in Methods. In (A), the cells were incubated for 15 min with increasing concentrations of agonists and cAMP production was then quantified. In (B), the cells were preincubated 15

min with a concentration of antagonists corresponding to ~10 fold K_i value as measured in binding experiments (see Table 1); increasing concentrations of 5-HT were then added for an additional 15 min before cAMP was measured. Values are expressed as the percentage of 5-HT maximal response. Each point is the mean of at least three independent experiments, each performed in triplicate. EC_{50} and E_{max} values are presented in Table 2.

Figure 4. Functional effects of potent 5-HT$_4$ receptor antagonists on basal and 5-HT-stimulated 5-HT$_{4(d)}$ receptors.

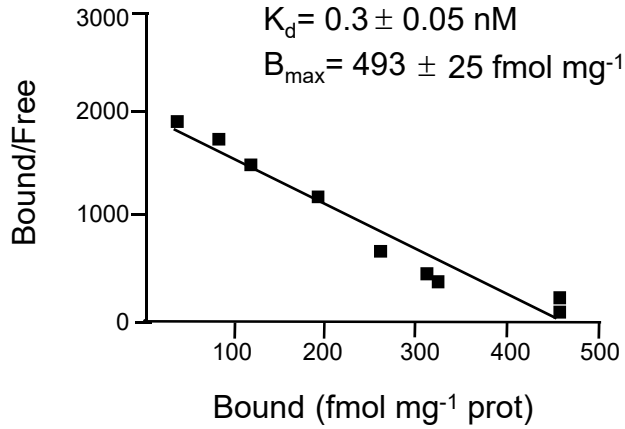
The effects of 5-HT, GR113808, ML10375, SB204070 and RS39604 in the absence or in the presence of 5-HT on cAMP production were determined in CHO cells expressing the h5-HT$_{4(d)}$ receptor. Results are expressed as percentage of control (untransfected CHO cells, UT). GR113808, ML10375, SB204070, RS39604 and 5-HT were used at a concentration of 1 μM. Values are mean ± S.E.M. of three independent experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 versus indicated values by *t*-test.

Figure 5. Concentration response curves of SB204070 and RS39604 on cAMP production in CHO cells expressing the h5-HT$_{4(d)}$ receptor.

cAMP measurements were performed on the h5-HT$_{4(d)}$ receptor as described in Materials and Methods. Values are expressed as the percentage of 5-HT maximal response. Neutral antagonist ML10375 (1 μM) was added 15 min before agonists. **A**, cells were incubated for 15 min with increasing concentrations of 5-HT (+) or SB204070 (■). EC_{50} and E_{max} values of SB204070 were 0.011±0.002 nM and 50.6±1.5 %, respectively. In the presence of ML10375 (1 μM) (□), EC_{50} and E_{max} values were 0.16±0.06 nM and 42±2 %, respectively. **B,** cells were incubated for 15 min with increasing concentrations of 5-HT (+) or RS39604 (●). EC_{50} and E_{max} values of RS39604 were 6.6±1.8 nM and 57.4±2.0 %, respectively. In the presence of

ML10375 (1 μM) (○), EC_{50} and E_{max} values were 807±472 nM and 54±6 %, respectively. Each point is the mean of at least three independent experiments, each performed in triplicate.

h5-HT$_{4(d)}$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B_{max}= 493 ± 25 fmol mg^{-1} prot
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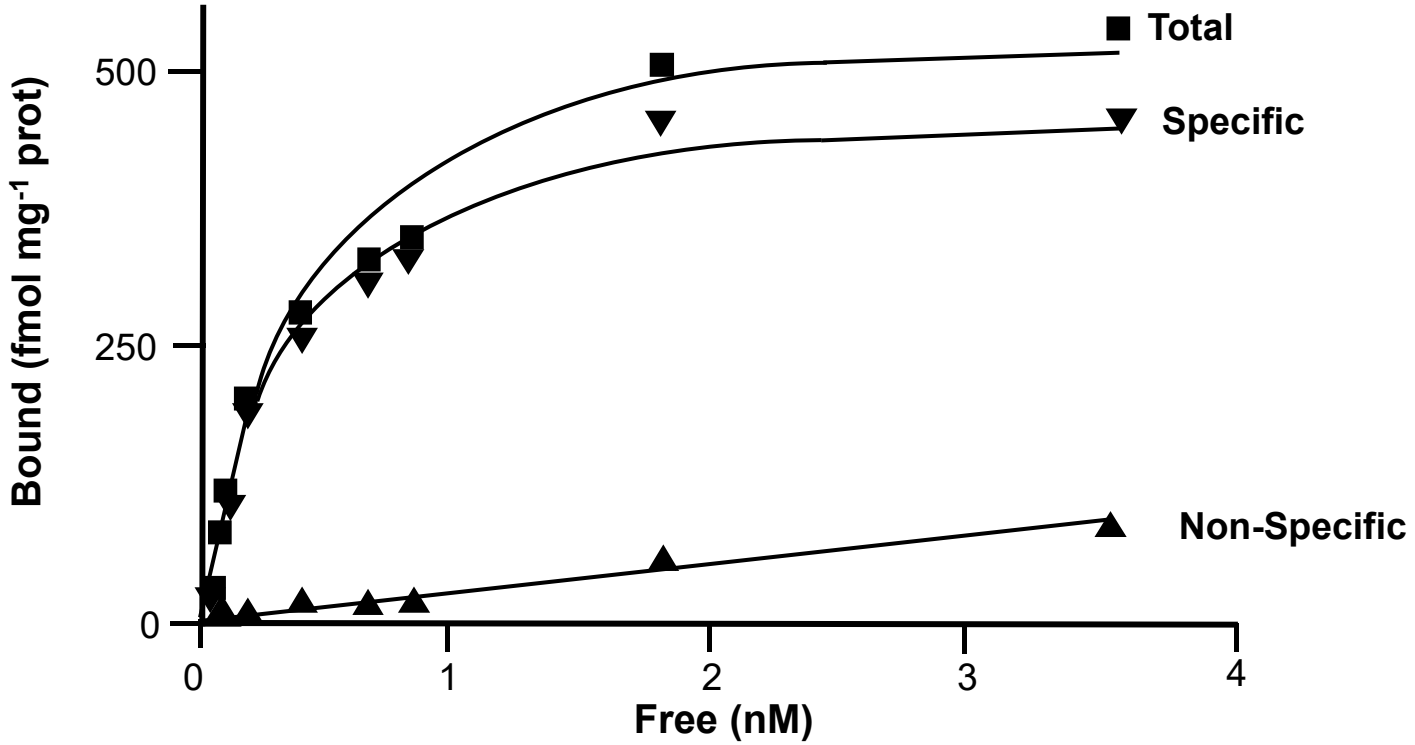
2000
1000
0
100
200
300
400
500
Bound (fmol mg^{-1} prot)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Specific
Non-Specific
Bound (fmol mg^{-1} prot)
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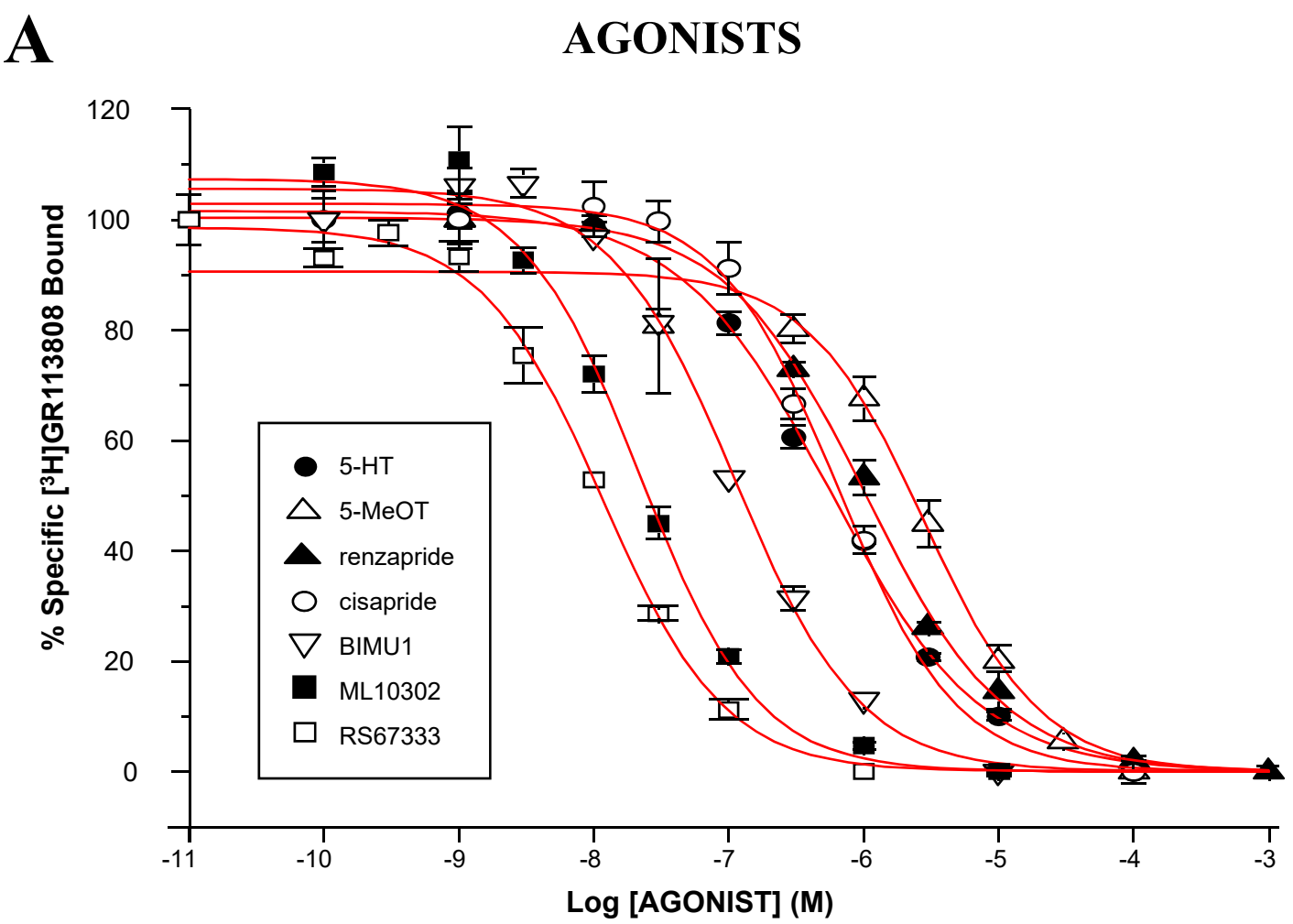
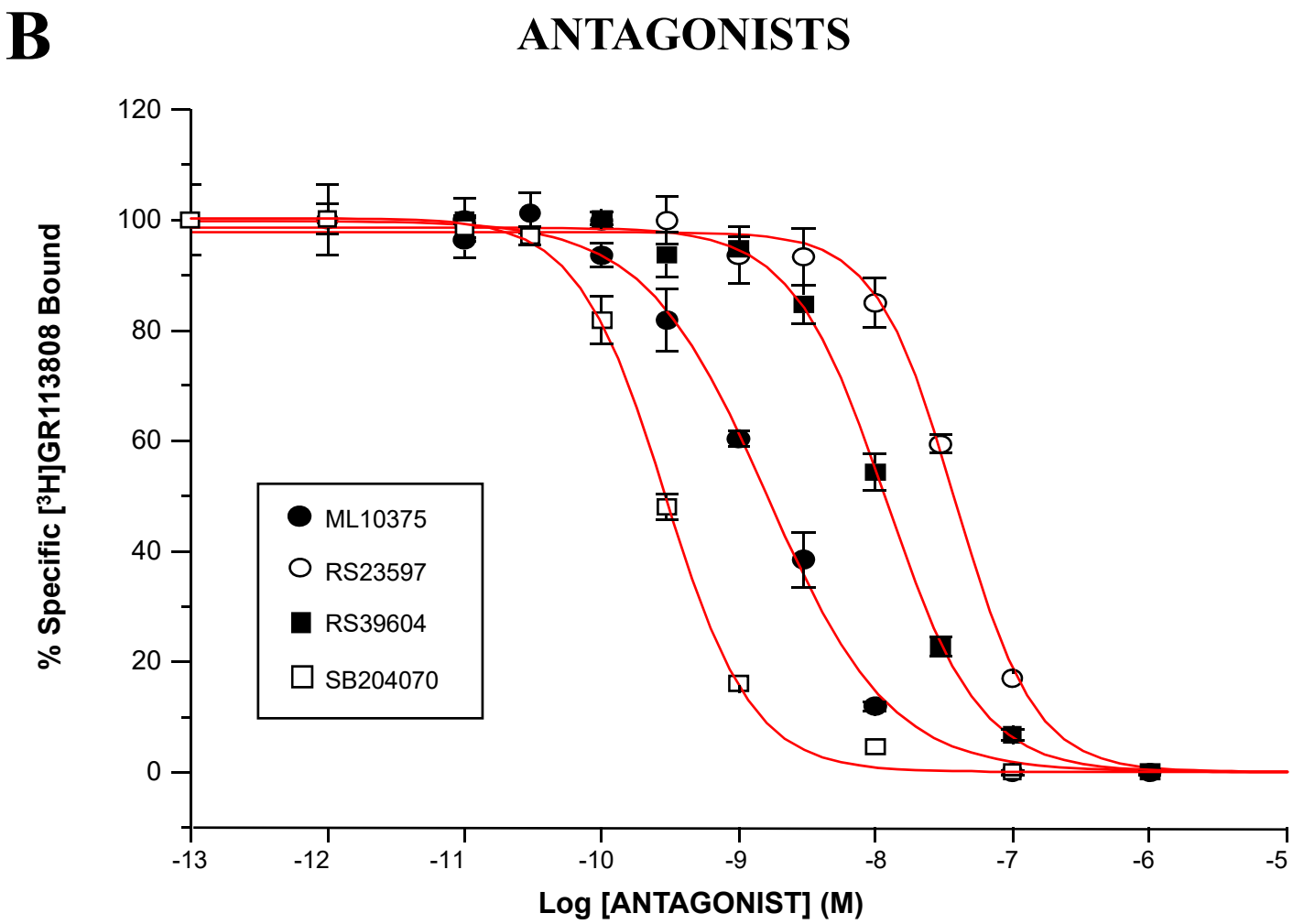
0
0
1
2
3
4
Free (nM)


A
AGONISTS
% Specific [³H]GR113808 Bound
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [AGONIST] (M)
5-HT
5-MeOT
renzapride
cisapride
BIMU1
ML10302
RS67333


B
ANTAGONISTS
% Specific [³H]GR113808 Bound
120
100
80
60
40
20
0
-13
-12
-11
-10
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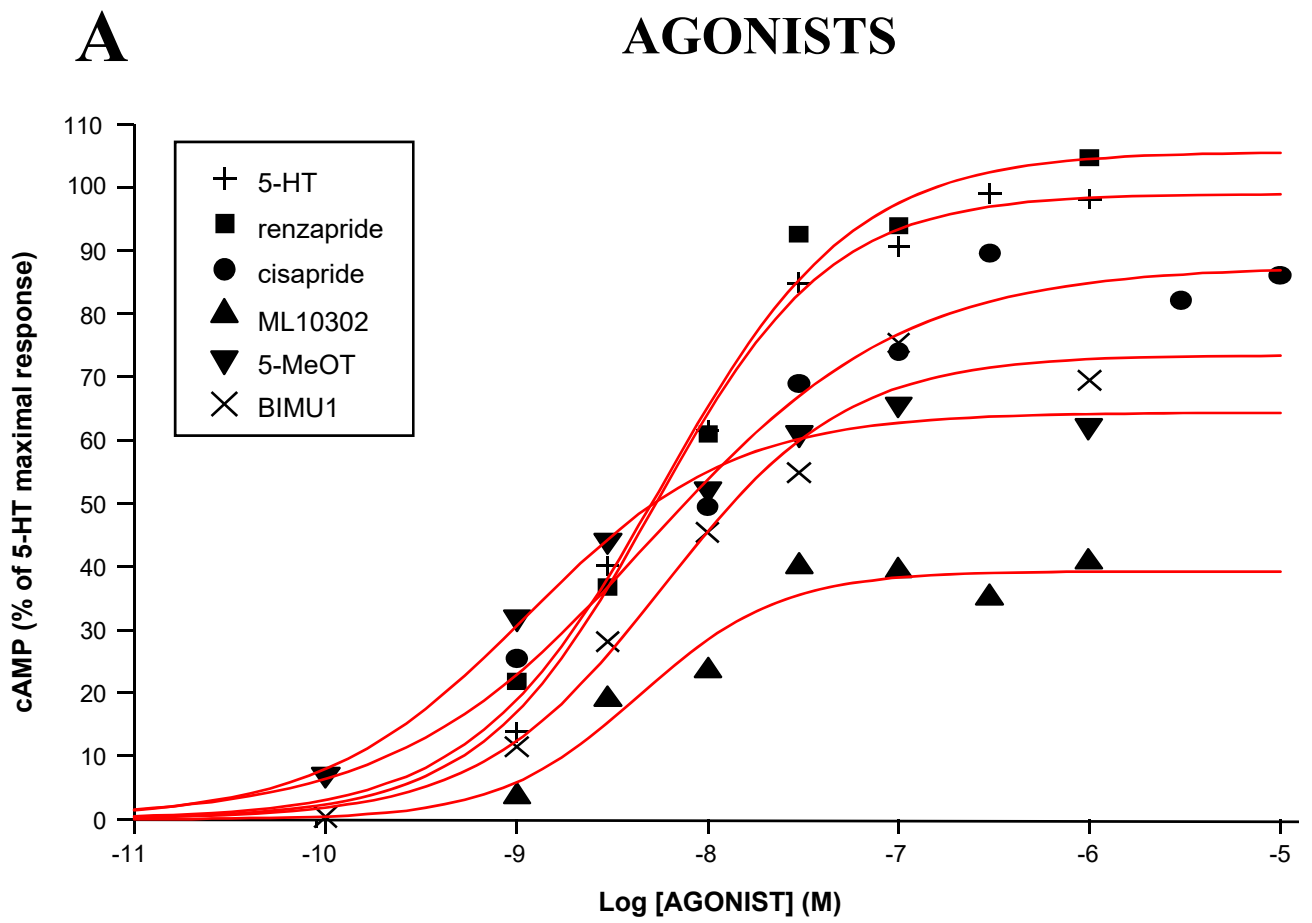
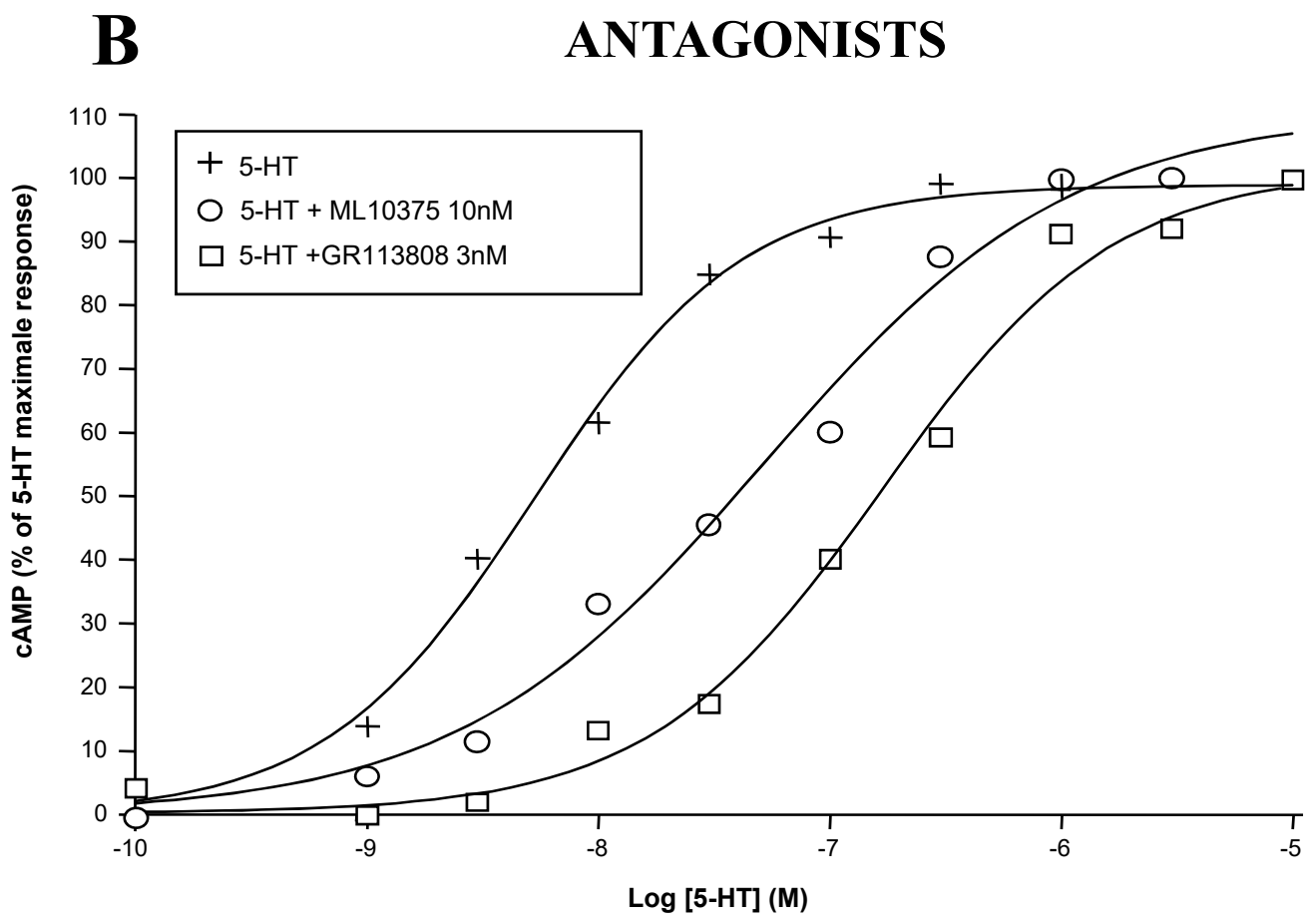
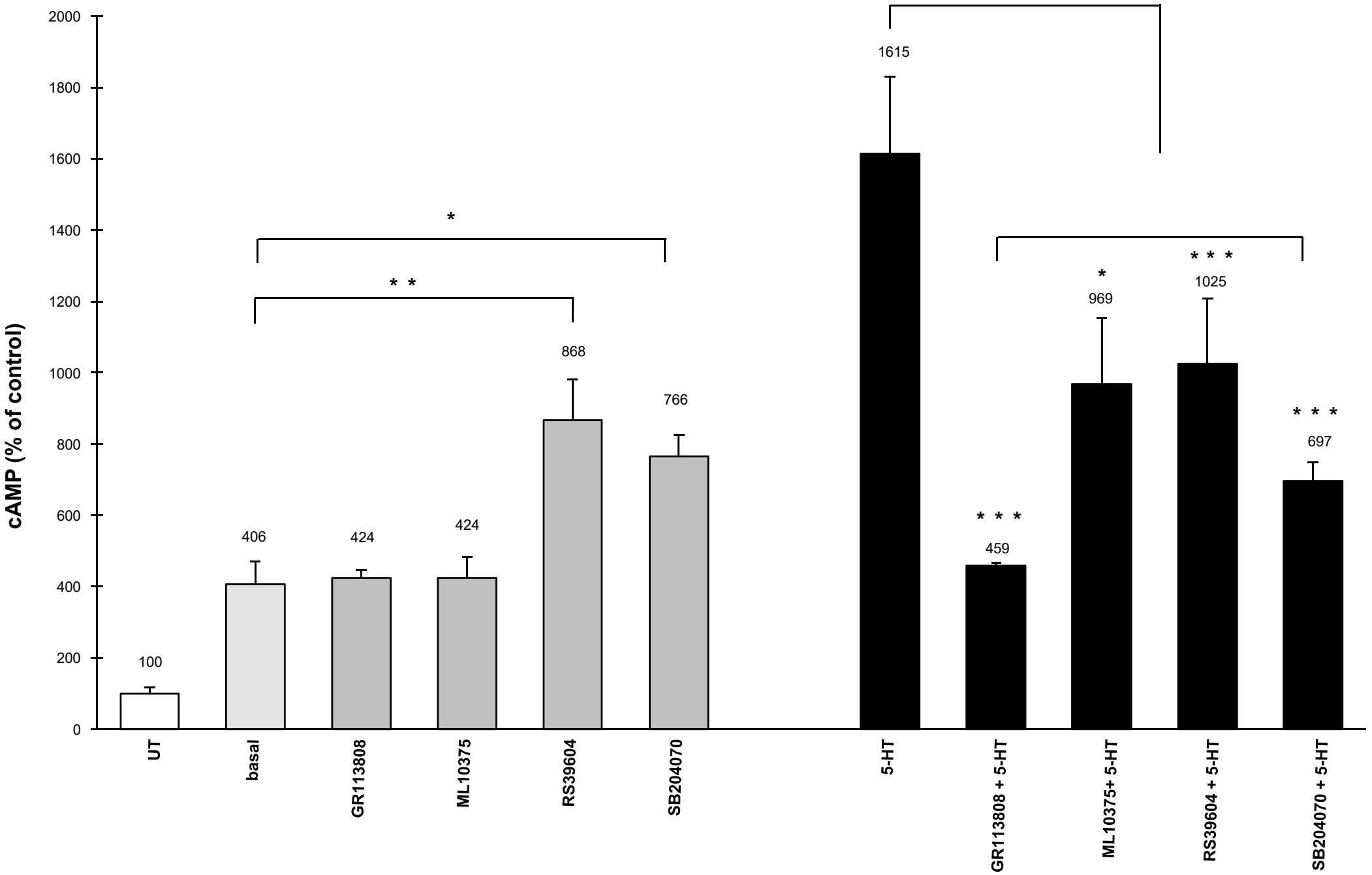
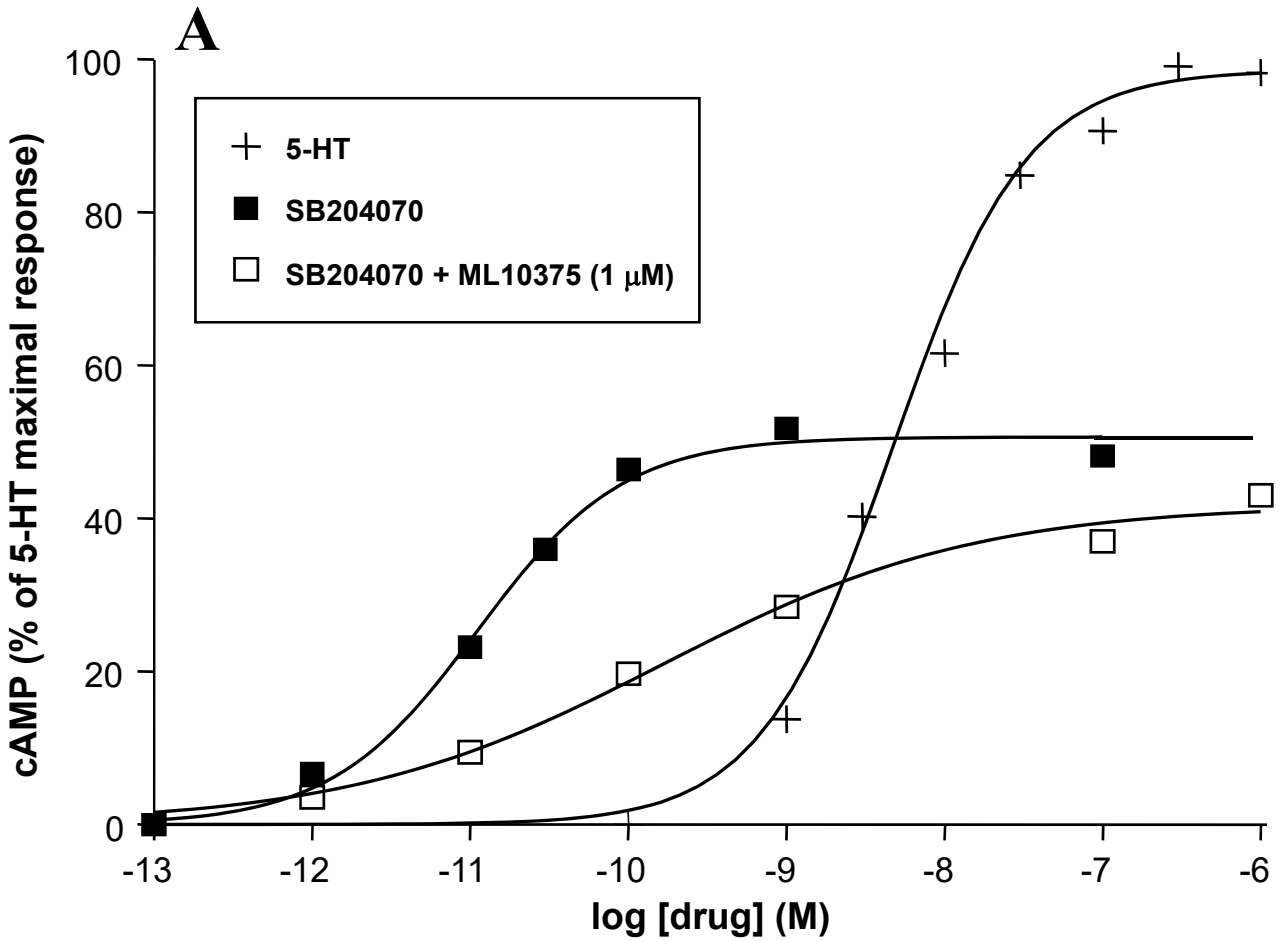
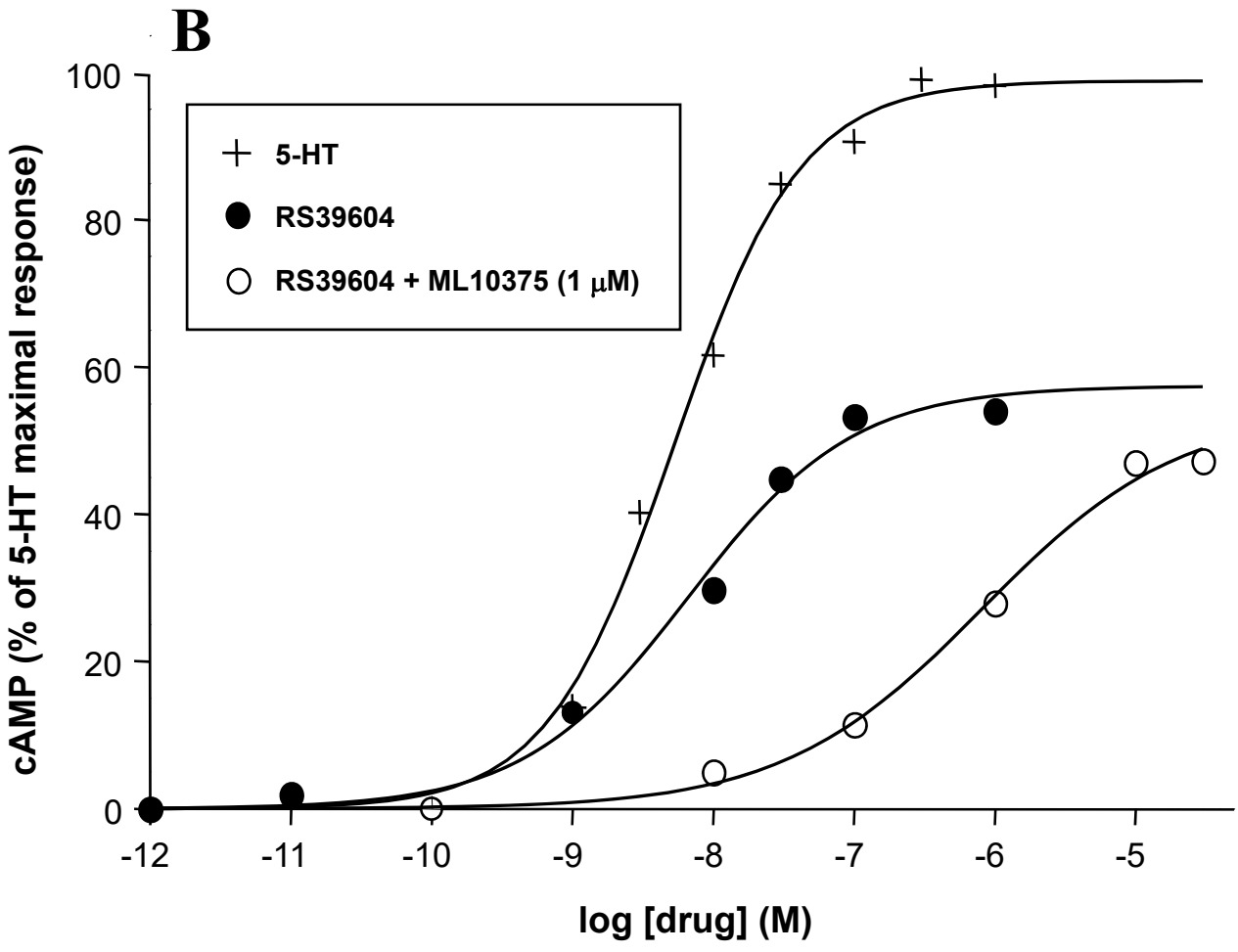
-7
-6
-5
Log [ANTAGONIST] (M)
ML10375
RS23597
RS39604
SB204070


A
AGONISTS
5-HT
renzapride
cisapride
ML10302
5-MeOT
BIMU1
cAMP (% of 5-HT maximal response)
Log [AGONIST] (M)


B
ANTAGONISTS
5-HT
5-HT + ML10375 10nM
5-HT +GR113808 3nM
cAMP (% of 5-HT maximale response)
Log [5-HT] (M)


cAMP (% of control)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
100
406
424
424
868
766
1615
459
969
1025
697
* * *
* *
*
UT
basal
GR113808
ML10375
RS39604
SB204070
5-HT
GR113808 + 5-HT
ML10375+ 5-HT
RS39604 + 5-HT
SB204070 + 5-HT


A
5-HT
SB204070
SB204070 + ML10375 (1 μM)
cAMP (% of 5-HT maximal response)
log [drug] (M)


B
5-HT
RS39604
RS39604 + ML10375 (1 μM)
cAMP (% of 5-HT maximal response)
log [drug] (M)