British Journal of Pharmacology (2007) 150, 782–791
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

A comparison of the pharmacological properties of guinea-pig and human recombinant 5-HT$_4$ receptors

RG Vickery[1], N Mai[1], E Kaufman[1], DT Beattie[2], T Pulido-Rios[2], M O'Keefe[1], PPA Humphrey[1] and JAM Smith[1]

[1]*Department of Molecular and Cell Biology, Theravance Inc., South San Francisco, CA, USA and* [2]*Department of Pharmacology, Theravance Inc., South San Francisco, CA, USA*

Background and purpose: 5-HT$_4$ receptor agonists are used therapeutically to treat disorders of reduced gastrointestinal motility. Since such compounds are evaluated in guinea-pigs, we cloned, expressed and pharmacologically characterized the guinea-pig 5-HT$_4$ and human 5-HT$_{4(b)}$ splice variant, which share 95% homology. The functional properties of guinea-pig 5-HT$_{4(b)}$ receptors were compared with native receptors in guinea-pig colon.
Experimental approach: Membrane radioligand binding and whole cell cAMP accumulation assays were used to determine the affinities, potencies and intrinsic activities (IA). Contraction of the guinea-pig distal colon longitudinal muscle myenteric plexus preparation (LMMP) was monitored to evaluate functional activity.
Key results: pK_i values for guinea-pig and human recombinant receptors, and guinea-pig striatum 5-HT$_4$ receptors, were in agreement, as were the potency and IA values for guinea-pig and human 5-HT$_4$ receptors expressed at a similar density ($\sim$0.2 pmol mg^{-1} protein). Tegaserod was a potent (pEC_{50} = 8.4 and 8.7, respectively), full agonist at both guinea-pig and human 5-HT$_4$ receptors. In contrast, in the LMMP preparation, tegaserod was a potent, partial agonist (pEC_{50} = 8.2; IA = 66%).
Conclusions and implications: Close agreement between the pharmacological properties of guinea-pig and human 5-HT$_4$ receptors support the use of guinea-pig model systems for the identification of 5-HT$_4$ receptor therapeutics. However, the mechanisms underlying the different agonist properties of tegaserod in recombinant and isolated tissue preparations, and the extent to which these impact the clinical efficacy of tegaserod as a prokinetic agent, remain to be determined.
British Journal of Pharmacology (2007) **150,** 782–791. doi:10.1038/sj.bjp.0707154; published online 12 February 2007

Keywords: gastrointestinal; 5-hydroxytryptamine; 5-HT$_4$; tegaserod; adenylate cyclase; gastroprokinetic

Abbreviations: DMEM, Dulbecco's modified Eagle medium; HEK293, human embryonic kidney 293; IA, intrinsic activity; LMMP, longitudinal muscle myenteric plexus; 5-MeOT, 5-methoxytryptamine

Introduction

The neurotransmitter 5-hydroxytryptamine (5-HT) mediates a broad range of physiological and behavioural responses both centrally and peripherally (Saxena, 1995; Barnes and Sharp, 1999). All classes of the extensive 5-HT receptor family, except for the ligand-gated 5-HT$_3$ receptor, are members of the seven transmembrane-spanning G protein-coupled family of receptors (Hoyer *et al*., 2002; Reeves and Lummis, 2002). These receptors modulate signal transduction pathways via stimulation or inhibition of adenylyl cyclase, modulation of cytosolic calcium concentration or activation of multiple alternative downstream effectors, including ERK1/2 (Pauwels, 2000; Norum *et al*., 2003).

The 5-HT$_4$ receptor is thought to signal principally, but not exclusively, via Gs coupling to activation of adenylyl cyclase (Gerald *et al*., 1995; Pindon *et al*., 2002). Peripherally, activation of sinoatrial 5-HT$_4$ receptors has a chronotropic effect on heart rhythm (Hegde and Eglen, 1996; Krobert *et al*., 2005; De Maeyer *et al*., 2006). In the digestive tract, activation of 5-HT$_4$ receptors initiates a coordinated and complex prokinetic response that has been extensively studied and well described (Gershon, 1999). 5-HT$_4$-mediated responses include modulation of cholinergic neurotransmission in the human proximal stomach (Leclere and Lefebvre, 2002) and increased colonic contractile responses in human, rat, dog and guinea-pig (Grider *et al*., 1998; Sakurai-Yamashita *et al*., 1999; Prins *et al*., 2000a, b; Leclere *et al*., 2005). Contraction and potentiation of the electrical field

Correspondence: Dr RG Vickery, Department of Molecular and Cell Biology, Theravance Inc., 901 Gateway Boulevard, South San Francisco, CA 94080, USA.
E-mail: rvickery@theravance.com
Received 4 September 2006; revised 22 November 2006; accepted 11 December 2006; published online 12 February 2007

twitch response in the guinea-pig ileum (Kajita *et al*., 2001) and colon (Jin *et al*., 1999) have also been described, as has facilitation of fast excitatory synaptic transmission (Galligan *et al*., 2003). In the rat oesophagus, 5-HT$_4$ receptors mediate relaxation of smooth muscle (Leung *et al*., 1996; Goldhill *et al*., 1997).

Direct activation of 5-HT$_4$ receptors has been demonstrated to relieve symptoms of several disease states, including functional bowel disorders, such as constipation-predominant irritable bowel syndrome and chronic constipation (Sanger, 1996; Alaradi and Barkin, 2002). Although the therapeutic potential of 5-HT$_4$ agonists in the treatment of these disorders is accepted (Emmanuel *et al*., 2002; Lacy and Yu, 2002; Camilleri, 2004; Johanson, 2004), opportunities still exist for the development of novel agents with improved efficacy and/or tolerability profiles. Preclinical evaluations of novel 5-HT$_4$ agonists include the use of human recombinant 5-HT$_4$ receptor systems (Hinschberger *et al*., 2003). However, these systems have a number of limitations. Thus, questions surrounding the influence of receptor expression levels (Krobert *et al*., 2005), identity of relevant signal transduction pathways (Pindon *et al*., 2002) and desensitization mechanisms (Barthet *et al*., 2005), for example, prompt evaluation of novel agents in isolated tissue preparations, in particular from dog (Prins *et al*., 2000a), guinea-pig (Beattie *et al*., 2004) and/or human (Leclere *et al*., 2005) digestive tract. Guinea-pig tissue is readily accessible and guinea-pig pharmacological models are used routinely for the evaluation of 5-HT$_4$ receptor selective agonists. Indeed, on the basis of work in guinea-pig isolated ileum, tegaserod has been described as a partial agonist (Buchheit *et al*., 1991). However, to date, although the guinea-pig 5-HT$_4$ receptor has been cloned, there has been no comprehensive evaluation of the pharmacological properties of this recombinant receptor. The aims of the present study were to characterize the pharmacological properties of the guinea-pig recombinant 5-HT$_4$ receptor in human embryonic kidney 293 (HEK293) cell stable transfectants, and compare these with native 5-HT$_4$ receptors. A two-step approach was undertaken, in which binding affinities at the recombinant 5-HT$_4$ receptor were compared with those at the native receptor in striatal membrane preparations, whereas functional comparisons were made with native guinea-pig 5-HT$_4$ receptors, using the guinea-pig isolated colon longitudinal muscle myenteric plexus (LMMP) preparation. Although guinea-pig striatum has been used extensively to determine ligand-binding affinities (e.g. Grossman *et al*., 1993), to the best of our knowledge, there are no *in vitro* functional studies using guinea-pig striatum. In contrast, the use of guinea-pig colon preparations to profile 5-HT$_4$ receptor ligands is well established (e.g. Leung *et al*., 1996). It should be noted, however, that there is an excellent correlation between the binding affinities for a range of agonist and antagonist ligands at 5-HT$_4$ receptors in striatal and ileal/colon membrane preparations (Uchiyama-Tsuyuki *et al*., 1996). In addition, in the present study, the properties of the guinea-pig 5-HT$_4$ receptor were compared directly with the prevalent human 5-HT$_{4(b)}$ receptor splice variant, with which it shares $\sim$95% identity, as well as high similarity within the C-terminus.

Methods

5-HT$_4$ receptor cloning and expression

Guinea-pig and human recombinant 5-HT$_4$ and 5-HT$_{4(b)}$ receptors were cloned by reverse transcription-polymerase chain reaction (RT-PCR) from guinea-pig striatum and a mixed ileum and jejunum human cDNA library, respectively. The sequence of the forward and reverse primers encoding the guinea-pig 5-HT$_4$ receptor were 5′-GTCTAGATGGA CAAACTTGATGCTAATGTGAG-3′ and 5′-CTCGAGTTACT AAGTGTCAATGGGCTGAGCAGCCACCAAAGGAGAACTTG CTGCAGGG-3′ and were designed using the published sequence provided in Genbank (van den Wyngaert *et al*., 1997, Genbank Accession Y13585). The forward and reverse primers encoding the human 5-HT$_{4(b)}$ receptor were 5′-ATG GACAAACTTGATGCTAATGTG-3′ and 5′-CTTCTGGGTCATT GTCCCAGG-3′, respectively. The final guinea-pig and human 5-HT$_4$ receptor constructs were fully sequenced in both the forward and reverse directions to confirm both the identity and the integrity of each sequence. 5-HT$_4$ receptor cDNA was subcloned into mammalian expression vectors and transfected into HEK293 cells by Ca^{2+}-phosphate-mediated uptake. Cells were grown in Dulbecco's modified Eagles medium (DMEM) and incubated in a 5% CO_2 humidified incubator at 37°C. Single colonies were generated by dilution cloning, under geneticin selection. Clones expressing 5-HT$_4$ receptors were identified using 5-HT$_4$ receptor selective agonists to stimulate adenylyl cyclase in a whole-cell cyclic adenosine monophosphate (cAMP) accumulation assay (see below). Corresponding receptor densities were determined in saturation-binding experiments using [^{3}H]GR113808, as described below. HEK293 cells stably transfected with guinea-pig 5-HT$_4$ or human 5-HT$_{4(b)}$ recombinant receptors, respectively, were cultured in DMEM supplemented with D-glucose (4500 mg l^{-1}), 10% foetal bovine serum and 100 U of penicillin-(100 μg) streptomycin ml^{-1} and geneticin (800 μg ml^{-1}) in a 5% CO_2, humidified incubator (37°C).

mRNA isolation and RT-PCR

Adult male, Dunkin-Hartley guinea-pigs (200–300 g, Harlan, Indiana) were killed by CO_2 asphyxiation and thoracotamy, in accordance with the Theravance Institutional Animal Care and Use Committee guidelines and the principles of laboratory animal care prescribed by the National Institute of Health. The oesophagus, duodenum, ileum, colonic submucosal plexus (SMP) and distal colon LMMP were harvested and stored in RNA*later* RNA Stabilization Reagent at −20°C, before mRNA extraction. Unless otherwise noted, all steps of the extraction were performed at 4°C. Tissue samples were normalized by weight following isolation and microdissection of segments from each region of the digestive tract. Tissue samples were predisrupted using a hand-held homogenizer and then homogenized using QIAshredder columns. Total RNA was isolated using the RNeasy Protect Mini Kit and treated with the DNA-*free* kit to remove any remaining genomic DNA. Guinea-pig 5-HT$_4$ cDNA was transcribed using the ThermoScript RT-PCR System and oligo dT primers in the presence of 3–5 μg RNA. PCR reactions were performed

in a final volume of 50 μl containing 100–500 ng cDNA template, 0.5 μM primers, 0.2 mM each deoxynucleoside triphosphate, 1 × PCR buffer, 1.5 mM $MgCl_2$ and 5 U Platinum *Taq* Polymerase. cDNA templates were denatured for 2 min at 95°C and amplified as follows: 95°C, 30 s; 55°C, 45 s; 72°C, 45 s; 42 cycles. A final extension was performed at 72°C for 7 min. The sequences of the forward and reverse primers encoding the C-terminus of the guinea-pig 5-HT$_4$ receptor were 5′-GCCTTCCTTATCATCCTGTGCTGTG-3′ and 5′-CTCGAGTTACTAAGTGTCAATGGGCTGAGCAGCCACCA AAG-3′, respectively. Amplified PCR products, generated over the linear range of amplification, were visualized on a 1.2% agarose gel.

Cell membrane preparation

At 20–22 h before harvest, HEK293 cells stably transfected with guinea-pig 5-HT$_4$ or human 5-HT$_{4(b)}$ receptor cDNA were washed twice and then cultured in serum-free DMEM. Cells were harvested by gentle mechanical agitation and then centrifugation (1200 *g*, 5 min). The pellets were resuspended in 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid) (HEPES), pH 7.4 and homogenized with a polytron disrupter (setting 19.2 × 10 s) on ice. The resultant homogenates were centrifuged (1200 *g*, 5 min), the pellets discarded and the supernatants centrifuged (40 000 *g* 20 min). The pellets were washed once by resuspension in 50 mM HEPES (pH 7.4) and centrifugation (40 000 *g* for 20 min). The final pellets were resuspended in 50 mM HEPES (pH 7.4) and aliquots stored at −80°C, until required.

Membranes were also prepared from guinea-pig striatum, ileum and proximal colon. Tissue pieces were harvested, as described above. Membranes were then prepared according to the protocol described for recombinant HEK293 cells.

5-HT$_4$ receptor radioligand binding

5-HT$_4$ receptor radioligand membrane binding assays were conducted as described previously (Bender *et al*., 2000). Briefly, membranes were incubated with [^{3}H]GR113808 in 50 mM Tris-HCl buffer pH.7.4. Nonspecific radioligand binding was defined using GR113808 (1 μM). Competition binding studies, to determine compound affinity, were conducted with increasing concentrations of unlabelled ligand (10 pM–30 μM) and at a fixed concentration of 0.15 nM [^{3}H]GR113808. Following a 60 min incubation at 22°C (a period sufficient to reach equilibrium), the membranes were harvested by rapid filtration over Whatman GF/B filters and bound radioactivity quantitated by liquid scintillation spectroscopy.

Whole-cell cAMP accumulation studies

Whole-cell cAMP accumulation studies were conducted using HEK293 cells stably transfected with guinea-pig 5-HT$_4$ or human 5-HT$_{4(b)}$ recombinant receptors, respectively, and the Flashplate Adenylyl Cyclase Activation Assay System, as described previously (Pindon *et al*., 2002).

Cells were cultured as described above. Cells were grown to 60–80% confluency and, at 20–22 h before harvest, washed twice and then cultured in serum-free DMEM. To harvest the cells, the media were aspirated and the cells were incubated for 5 min at room temperature with Versene. The cells were lifted from the flask by gentle mechanical agitation, suspended in pre-warmed (37°C) Dulbecco's phosphate-buffered saline, and then harvested by centrifugation at 1200 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in pre-warmed (37°C) 'stimulation buffer' provided with the Flashplate kit. Cells were diluted to a concentration of 5×10^5 cells ml^{-1} in pre-warmed (37°C) 'stimulation buffer' and preincubated at 37°C for 10 min. cAMP accumulation assays were performed with increasing concentrations of test compound and 5-HT (10 pM–100 μM). Cell suspension (50 μl) was added to each well of the Flashplate for a final assay volume of 100 μl. The cells were incubated, with shaking, at 37°C for 15 min. After the incubation period, a direct radioimmunoassay, using [^{125}I] cAMP, was performed by the addition of 100 μl of ice-cold 'detection buffer' to each well, according to the manufacturer's instructions. The plates were sealed and incubated at 4°C, overnight. Bound radioactivity was quantified by scintillation proximity spectroscopy using the Topcount (Perkin-Elmer, Boston, MA, USA). The amount of cAMP produced was extrapolated from a cAMP standard curve.

Guinea-pig isolated distal colon LMMP preparation

Adult, male, Dunkin-Hartley guinea-pigs (200–300 g, Harlan, Indiana) were killed by CO_2 asphyxiation and thoracotamy, in accordance with the Theravance Institutional Animal Care and Use Committee guidelines. The colon was removed, and placed in Krebs–Henseleit physiological buffer, containing (in mM): KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ anhydrous 1.2, NaCl 118.1, D-glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2$ 2.6, ondansetron 0.003 (to block 5-HT$_3$ receptors), methysergide 0.001 (to block 5-HT$_1$ and 5-HT$_2$ receptors) and indomethacin 0.001 (to inhibit prostaglandin synthesis). The colon was cut into 5 cm lengths, the contents gently removed and each segment then placed on a 10 ml pipette. An incision was made along the length of the colon with a scalpel blade, and the longitudinal muscle was then peeled off carefully using the tip of a thumb. Each longitudinal muscle strip was then mounted, under a tension of 1 g, in a 10 ml tissue bath filled with Krebs–Henseleit buffer. The bathing solution was aerated continuously with 95% O_2/5% CO_2 and maintained at 37°C. During the next 45 min, tissues were washed three times (at 0, 15 and 30 min after mounting) and tension re-applied to 1 g as necessary. The tissues were then challenged with 5-HT at a concentration (0.3 μM) previously established to evoke a maximal contractile response. Once the contraction had reached its maximum, tissues were washed four times every 2 min and once more 10 min later. An additional priming challenge of 5-HT (0.3 μM) was made 15 min later. Following further washing, before and after a third 5-HT (0.3 μM) challenge to confirm the maximal contractile response had been determined, a cumulative concentration–effect curve to 5-HT (0.0001–3 μM) or test compound (0.0001–10 μM) was constructed. Responses were allowed to reach a maximum before addition of the next, ascending concentration. Contractile responses were normalized to the primed 5-HT (0.3 μM) response in each tissue.

Data analysis

Binding data were analysed by nonlinear regression analysis using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) and a three-parameter model for one-site competition. pK_i (negative decadic logarithm of K_i) values for test compounds were calculated from the best-fit IC_{50} values, and the K_d value of the radioligand, using the Cheng-Prusoff equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1 + [L]/K_d)$ where [L] is concentration radioligand. Data are expressed as mean ± s.d. cAMP accumulation data were analysed by nonlinear regression analysis with the GraphPad Prism Software package using the three-parameter sigmoidal dose-effect model (slope constrained to unity). Potency data are presented as pEC_{50} values (negative decadic logarithm of the effective concentration producing 50% of the maximum response; mean ± s.e.m.). To determine the IA, relative to 5-HT, the maximum compound-evoked response (minus basal) was expressed as a percentage of the maximum response evoked by 5-HT (minus basal), assayed in parallel on the same plate. LMMP contractile responses were analysed by nonlinear regression analysis with GraphPad Prism Software using the three-parameter sigmoidal dose–effect model (slope constrained to unity) to derive the mean potency (pEC_{50} ± s.e.m.) and IA (as a percentage of the 5-HT maximum response).

Materials

The mixed ileum and jejunum human cDNA library was purchased from Clontech (Mountain View, CA, USA). Tissue culture reagents, ThermoScript RT-PCR System and *taq* polymerase were purchased from Invitrogen (Carlsbad, CA, USA). The RNA*later* RNA Stabilization Reagent, QIAshredder columns and the RNeasy Protect Mini Kit were purchased from Qiagen (Valencia, CA, USA). The DNA-*free* kit was purchased from Ambion (Austin TX, USA). HEPES, Tris-HCl, KCl KH_2PO_4, $MgSO_4$, NaCl, D-glucose, $NaHCO_3$, $CaCl_2$, 5-HT and 5-methoxytryptamine (5-MeOT) were purchased from Sigma-Aldrich (St Louis, MO, USA). [^{3}H]GR113808 was purchased from Amersham Biosciences (Newark, NJ, USA). The Flashplate Adenylyl Cyclase Activation Assay System was purchased from Perkin-Elmer (Boston MA, USA). Tegaserod and ondansetron were purchased from Apin Chemicals and Sequoia Research Products (Oxfordshire, UK), respectively. GR113808 ([1-2[(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl-1-methyl-1*H*-indole-3-carboxylate), RS39604 (1-[4-amino-5-chloro-2-(3,5-dimethoxybenzyloxy)phenyl]-3-[1-[2-[(methylsulphonyl)amino]ethyl]-4-piperidinyl]-1-propanone hydrochloride), SB203186 (1-piperidinylethyl1H-indole-3-carboxylate), RS67506 (1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-*n*-butyl-4-piperidinyl)-1-propanone, RS67333 (1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1-butyl-4-piperidinyl]-1-propanone), RS23597-190 (3-(piperdine-1-yl)-propyl-4-amino-5-chloro-2-methoxy benzoate hydrochloride), RS56812 (*N*-(quinuclidin-3-yl)-2-(1-methyl-1*H*-indol-3-yl)-2-oxoacetamide), methysergide and indomethacin were purchased from Tocris Cookson (Ellisville, MO, USA). Piboserod, TS951 (*N*-[endo-8-(3-hydroxypropyl)-8-azabicyclo[3.2.1]oct-3-yl]-1-isopropyl-2-oxo-1,2-dihydro-3-quinolinecarboxamide), prucalopride, cisapride, BIMU-8 (endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl)ethyl-2-oxo-1H-benzimidazole-carboxamide HCl), mosapride, ML10302 (2-(1-piperidinyl)ethyl-4-amino-5-chloro-2-methoxybenzoate) and metoclopramide were prepared by the Theravance Inc. Medicinal Chemistry department for research purposes.

For radioligand-binding and cAMP accumulation studies, 5-HT$_4$ ligands were prepared at 10 mM in dimethyl sulfoxide (DMSO), diluted to 400 μM with 50 mM HEPES (pH 7.4) at 25°C, containing 0.1% bovine serum albumin, and then serially diluted in the same buffer. For examining contractile responses in the LMMP preparation compounds were prepared at 10 mM in DMSO and then serially diluted in water.

Results

Distribution of guinea-pig 5-HT$_4$ receptors in the digestive tract

A guinea-pig 5-HT$_4$ receptor variant was cloned by RT-PCR from guinea-pig striatum. DNA sequence analysis confirmed the identity of this receptor and that the sequence shared the highest C-terminal identity with the human 5-HT$_{4(b)}$ receptor.

Using RT-PCR, the distribution pattern of this 5-HT$_4$ receptor variant was examined in various tissue regions isolated from the guinea-pig digestive tract. Guinea-pig 5-HT$_4$ receptor mRNA transcripts were detected in all regions examined, specifically the duodenum, ileum, oesophagus, LMMP and colonic SMP (Figure 1). The apparent highest and lowest levels of 5-HT$_4$ receptor mRNA were detected in the duodenum and oesophagus, respectively.

The presence of saturable, specific [^{3}H]GR113808 binding sites in membranes prepared from guinea-pig isolated ileum and proximal colon, respectively, confirmed the presence of 5-HT$_4$ receptor protein in these tissues. [^{3}H]GR113808 bound to a single population of sites in both ileum and colon with pK_D values of 10.6 and 10.4 and corresponding B_{max} values of 37 and 28 fmol mg^{-1} protein, respectively ($n = 2$). For comparison, the pK_D and B_{max} values for [^{3}H]GR113808 were 10.31 ± 0.14 and 147 ± 26 fmol mg^{-1} protein ($n = 6$), respectively, in membranes prepared from guinea-pig striatum.

Pharmacological characterization of guinea-pig recombinant 5-HT$_4$ receptors

Stable transfectants expressing 5-HT$_4$ receptors at both low (gp 5-HT$_4$-clone 1, $B_{max} = 0.21 \pm 0.03$ pmol mg^{-1} protein) and high (gp 5-HT$_4$-clone 2, $B_{max} = 2.54 \pm 1.18$ pmol mg^{-1}

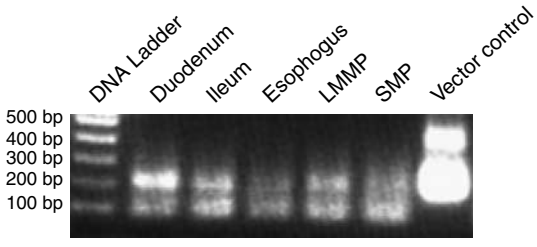

Figure 1 Analysis of 5-HT$_4$ receptor cDNA fragments isolated from representative regions of guinea-pig digestive tract. The DNA markers are shown in the far left lane. The amplified fragments corresponding to the C-terminus of the 5-HT$_4$ receptor can be visualized as the upper 0.2 kb band.

protein) receptor densities were used for pharmacological studies. [^{3}H]GR113808 bound with high affinity (pK_D = 10±0.48 and 10.18±0.29, respectively), to a single population of sites in membranes prepared from both gp 5-HT$_4$-clone 1 and gp 5-HT$_4$-clone 2.

The B_{max} value in the gp 5-HT$_4$ – clone 1 was similar to that observed in guinea-pig striatum. Therefore, membranes prepared from this clone were used in [^{3}H]GR113808 competition binding studies to profile the binding affinities of a panel of 5-HT$_4$ receptor selective agonists and antagonists. As can be seen from Table 1, 5-HT$_4$ receptor selective antagonists, for example GR113808 and SB203186, bound with high affinity ($pK_i \geqslant 9.9$) to guinea-pig recombinant 5-HT$_4$ receptors. The 5-HT$_4$ receptor selective agonists, for example tegaserod, TS-951 and cisapride, bound with medium to high affinity ($pK_i \geqslant 7.7$), whereas metoclopramide and 5-MeOT exhibited low affinity ($pK_i \leqslant 6.9$).

The functional properties of guinea-pig recombinant 5-HT$_4$ receptors were characterized using whole-cell cAMP accumulation studies. To examine the influence of receptor density on the potency and IA of a range of 5-HT$_4$ receptor selective ligands, these studies were conducted using both gp 5-HT$_4$ – clone 1 and gp 5-HT$_4$ – clone 2.

Basal cAMP accumulation in 5-HT$_4$ transfectants (gp 5-HT$_4$ – clone 1 and gp 5-HT$_4$ – clone 2) and untransfected HEK293 cells was not significantly different (data not shown). 5-HT evoked a concentration-dependent increase in whole-cell cAMP accumulation in both gp 5-HT$_4$ – clone 1 and gp 5-HT$_4$ – clone 2 (Figure 2a) with corresponding pEC_{50} values of 7.9±0.3 (n=11) and 9.0±0.3 (n=5), respectively (Table 2). A similar increase in potency of one log unit, or greater, was observed for all 5-HT$_4$ receptor agonists in gp 5-HT$_4$ – clone 2, relative to gp 5-HT$_4$ – clone 1 (Table 2, Figure 2b–e). However, the rank order of potency was unchanged and, was as follows: tegaserod > 5-HT > cisapride > mosapride > metoclopramide (Table 2). The rank order of IA (expressed as a percentage of the maximal 5-HT response) in the lower receptor density clone, gp 5-HT$_4$ – clone 1, was as follows: tegaserod (106%) > cisapride (76%) ⩾ prucalopride (66%) > metoclopramide (24%) > mosapride (22%) (Table 2). In comparison, all the compounds tested were full agonists (IA > 90%) in the higher receptor density clone, gp 5-HT$_4$ – clone 2 (Table 2, Figure 2a–e). Notably, metoclopramide and mosapride were weak partial agonists (IA < 24%) in the lower receptor density clone, gp 5-HT$_4$ – clone 1, but behaved as full agonists in the higher receptor density clone, gp 5-HT$_4$ – clone 2 (Figure 2d and e, respectively).

Comparison between the pharmacological properties of guinea-pig recombinant and native 5-HT$_4$ receptors

[^{3}H]GR113808 radioligand membrane binding and conventional isolated tissue bath methodologies were used to characterize the pharmacological properties of guinea-pig native 5-HT$_4$ receptors. The binding affinities of a panel of 5-HT$_4$ receptor selective agonists and antagonists for guinea-pig native 5-HT$_4$ receptors in striatal membranes were in very close agreement (r^2 = 0.95, slope = 0.92±0.05; P < 0.0001) with the corresponding values for guinea-pig recombinant 5-HT$_4$ receptors (Table 1; Figure 3).

Table 1 Binding affinities (pK_i) for a range of 5-HT$_4$ ligands at native 5-HT$_4$ receptors in guinea-pig striatal membranes and recombinant guinea-pig 5-HT$_4$ and human 5-HT$_{4(b)}$ receptors stably expressed in HEK293 cells

Compound	*gp 5-HT$_4$/HEK293*	*gp striatal membranes*	*h 5-HT$_{4(b)}$/HEK293*
	pK$_i$ *(mean±s.d.)*	pK$_i$ *(mean±s.d.)*	pK$_i$ *(mean±s.d.)*
GR113808	10.3±0.2	10.4±0.1	10.5±0.1
RS39604	10.1±0.5	9.8±0.1	10.1±0.1
SB203186	9.9±0.2	9.3±0.2	9.4±0.1
RS67506	9.4±0.1	8.9±0.1	9.0±0.1
RS67333	8.7±0.1	8.7±0.1	9.0±0.1
RS23597–190	8.6±0.1	8.3±0.1	ND
Tegaserod	8.4±0.2	7.7±0.3	8.1±0.1
TS951	8.1±0.1	7.5±0.2	7.6±0.1
Prucalopride	ND	7.7±0.1	7.5±0.1
Cisapride	7.7±0.1	7.4±0.2	7.4±0.1
BIMU-8	7.6±0.2	6.9±0.2	7.2±0.1
Mosapride	7.4±0.2	7.0±0.1	7.2±0.1
5-HT	7.4±0.2	7.2±0.2	6.9±0.2
RS56812	7.2±0.1	6.7±0.2	7.1±0.1
5-MeOT	6.9±0.1	6.4±0.1	6.4±0.1
Metoclopramide	6.7±0.2	6.3±0.4	6.7±0.1

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-MeOT, 5-methoxytryptamine; ND, not determined.
Competition binding experiments were as described in the Methods section. Data are given as the mean±s.d. from at least three independent experiments.

The functional activity of guinea-pig native 5-HT$_4$ receptors was evaluated using the guinea-pig isolated distal colon LMMP preparation. The guinea-pig isolated colon LMMP preparation has been used widely as a bioassay for 5-HT$_4$ agonist and antagonist activity (Wardle and Sanger, 1993; Leung *et al*., 1996; Beattie *et al*., 2004). 5-HT, and a panel of 5-HT$_4$ receptor selective agonists, produced a concentration-dependent contraction of the LMMP preparation, with a range of potency and IA values (Figure 4a, Table 2). The rank order of potency (pEC_{50}) for this panel of compounds was as follows: tegaserod (8.2) ⩾ 5-HT (8.0) > prucalopride (7.6) > cisapride (7.0) > metoclopramide (5.5) = mosapride (5.4). The rank order of IA values, relative to 5-HT, was as follows: prucalopride (93%) > metoclopramide (84%) > cisapride (74%) > tegaserod (66%) > mosapride (37%). The contribution of 5-HT$_4$ receptor activation to tegaserod-evoked responses in the guinea-pig distal colon LMMP preparation was confirmed by pretreatment with the 5-HT$_4$ receptor selective antagonist piboserod. Piboserod attenuated tegaserod-evoked contractions with an apparent pK_b = 9.09 (n=2, 9.05 and 9.12).

Comparison between the pharmacological properties of guinea-pig and human recombinant 5-HT$_4$ receptors

There is 95% similarity between the sequences of the human 5-HT$_4$ receptors and the guinea-pig 5-HT$_4$ receptor cloned in this study, up to, but not including, the divergent C-terminus described for the human 5-HT$_4$ receptor isoforms. The significant similarity, 93% between the C-terminus (from amino acid 358 onwards), of the guinea-pig 5-HT$_4$ receptor described here and the human 5-HT$_{4(b)}$

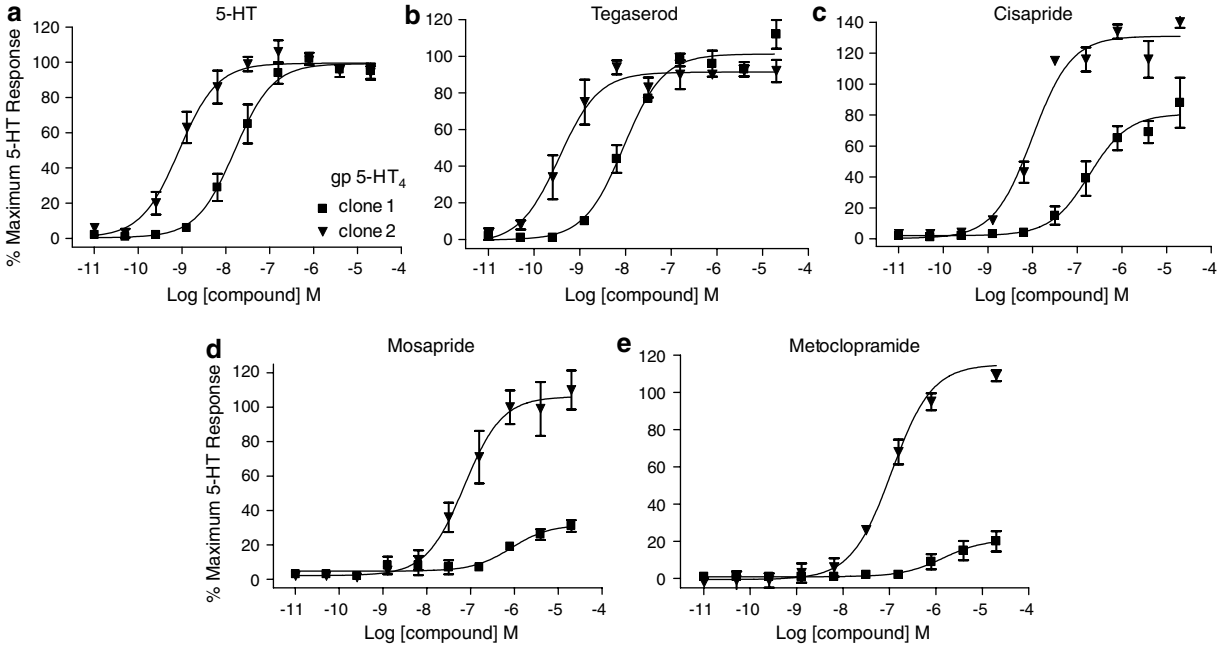

Figure 2 Agonist-evoked cAMP accumulation in HEK293 cells expressing 5-HT$_4$ receptors at a low (clone 1) and high (clone 2) receptor density, respectively. Data points (mean and s.e.m. (vertical lines)) from at least three separate experiments were used to generate curves for 5-HT (**a**), tegaserod (**b**), cisapride (**c**), mosapride (**d**) and metoclopramide (**e**) that were normalized to the maximum 5-HT-evoked response.

Table 2 The mean potency (pEC_{50}) and IA values determined in cAMP accumulation assays for guinea-pig 5-HT$_4$ and the human 5-HT$_{4(b)}$ receptors expressed in HEK293 cells and in the guinea-pig isolated colon LMMP preparation

Compound	*gp 5-HT$_4$ /HEK293 – clone 1*			*gp 5-HT$_4$/HEK293 – clone 2*			*h 5-HT $_{(4b)}$ /HEK293*			*gp isolated LMMP*		
	PEC_{50} mean±s.e.m.	*IA mean (95% CI)*	n	*pEC_{50} Mean±s.e.m.*	*IA mean (95% CI)*	n	*pEC_{50} Mean±s.e.m.*	*IA mean (95% CI)*	n	*pEC_{50} Mean±s.e.m.*	*IA mean (95% CI)*	n
Tegaserod	8.4±0.1	106 (99–111)	11	9.4±0.1	95 (99–111)	5	8.7±0.1	127 (107–135)	6	8.2±0.1	66 (61–72)	3
Prucalopride	7.9±0.1	66 (25–107)	3	ND	ND		8.2±0.1	95 (88–102)	6	7.5±0.1	93 (87–100)	14
5-HT	7.9±0.1	100	11	9.0±0.1	100	5	8.4±0.1	100	6	8.0±0.1	98 (94–102)	20
Cisapride	6.9±0.1	76 (63–89)	11	8.0±0.1	121 (104–138)	5	7.3±0.1	102 (92–107)	6	7.0±0.1	74 (69–80)	10
Mosapride	6.3±0.1	22 (15–30)	11	7.1±0.1	105 (85–125)	5	6.7±0.1	35 (24–47)	6	5.4±0.1	37 (32–42)	3
Metoclopramide	5.9±0.2	24 (15–32)	6	7.0±0.2	90 (78–102)	5	5.9±0.1	39 (0–78)	3	5.5±0.1	84 (76–92)	8

Abbreviations: CI, confidence interval; 5-HT, 5-hydroxytryptamine; HEK293, human embryonic kidney 293; IA, intrinsic activity; LMMP, longitudinal muscle myenteric plexus; ND, not determined.
Values represent mean±s.e.m. or 95% CI and the number of individual experiments.

receptor splice variant, suggests that the 5-HT$_{4(b)}$ receptor represents the human orthologue. Therefore, the pharmacological properties of guinea-pig 5-HT$_4$ and human 5-HT$_{4(b)}$ recombinant receptors, both stably expressed in HEK293 cells, were compared directly.

The binding affinities of a panel of 5-HT$_4$ receptor selective agonists and antagonists for human 5-HT$_{4(b)}$ (B_{max} = 0.34±0.03 pmol mg^{-1} protein) and guinea-pig 5-HT$_4$ recombinant receptors were in very close agreement (r^2 = 0.96, slope = 1.06±0.06; P<0.0001) (Table 1; Figure 5a). Furthermore, there was a very good correlation between the potency (r^2 = 0.99, slope = 0.89±0.03; P<0.0001) (Table 2; Figure 5b) and IA (r^2 = 0.91, slope = 0.85±0.1; P<0.0001) (Table 2; Figure 5c) values of a range of 5-HT$_4$ receptor selective ligands for the elevation of cAMP levels in guinea-pig 5-HT$_4$ and human 5-HT$_{4(b)}$ receptor transfectants.

Discussion

The present study has demonstrated that the pharmacological properties of guinea-pig native and recombinant 5-HT$_4$ receptors are very similar. Thus, there was a strong correlation between the binding affinities for a range of 5-HT$_4$ receptor selective agonists and antagonists at 5-HT$_4$ receptors in guinea-pig striatal membranes and the guinea-pig recombinant 5-HT$_4$ receptor. Furthermore, the potency values of a panel of 5-HT$_4$ receptor selective agonists for contraction of guinea-pig isolated colon LMMP preparations and in whole-cell cAMP accumulation assays, using HEK293 cells stably transfected with the recombinant receptor, were similar. The results of the present study confirm that 5-HT$_4$ receptor density can influence the apparent potency and IA of 5-HT$_4$ receptor agonists, as has been reported previously for other

classes of G-protein-coupled receptor (Kenakin, 2002). These observations emphasize the importance of characterizing the functional activity of compounds of interest in recombinant systems expressing 5-HT$_4$ receptors at densities that are believed to best approximate physiological levels. By extension, these studies also highlight the value of profiling at least subsets of compounds in relevant target tissues, whenever possible.

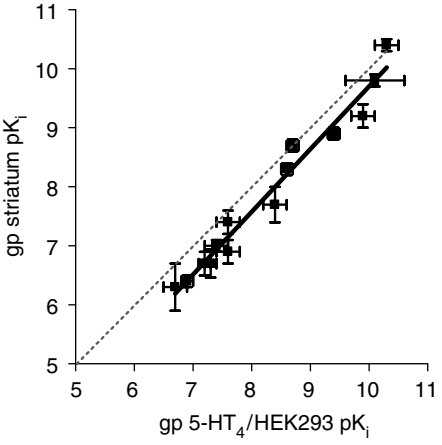

Figure 3 Correlation plot for a range of 5-HT$_4$ receptor agonist and antagonist pK_i values at recombinant and native guinea-pig 5-HT$_4$ receptors determined using [^{3}H]GR113808 competition binding assays. The solid line represents a linear regression through the points, and the dashed line represents unity. Data represent mean ± s.d. of 3–12 independent experiments.

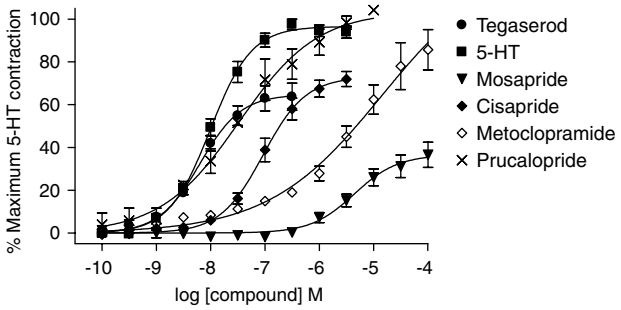

Figure 4 Concentration–response curves to tegaserod, 5-HT, prucalopride, cisapride, metoclopramide and mosapride in the guinea-pig isolated colon LMMP. Values are expressed as the mean ± s.e.m (vertical lines) change in tension, as a percentage of the primed, maximum 5-HT (0.3 μM) response in the same tissue.

A wide variety of methodologies, ranging from functional analyses in isolated tissue preparations to immunohistochemical studies, have been used to describe the distribution of 5-HT$_4$ receptors in mouse, rat, guinea-pig, dog and human digestive tract (Cohen *et al*., 1994; Sakurai-Yamashita *et al*., 1999; Prins *et al*., 2000a; Liu *et al*., 2005). In the present study, 5-HT$_4$ mRNA transcripts were found ubiquitously in the guinea-pig digestive tract. The corresponding density of [^{3}H]GR113808 binding sites in the ileum and proximal colon was $\sim$30 fmol mg^{-1} protein. This is consistent with values of 45 (Yoshikawa *et al*., 1998) or 12 (Uchiyama-Tsuyuki *et al*., 1996) fmol mg^{-1} protein, obtained previously for [^{3}H]GR113808 binding to guinea-pig ileum. In humans, there has been no direct measurement of 5-HT$_4$ receptor densities in the digestive tract. However, using autoradiographic techniques, 5-HT$_4$ receptor binding sites have been identified in both the myenteric plexus and muscle layers of human colon (Sakurai-Yamashita *et al*., 1999). Interestingly, in the present study, 5-HT$_4$ receptor mRNA transcripts were detected in guinea-pig oesophageal tissue, consistent with recent studies demonstrating 5-HT$_4$ receptor immunoreactivity in the muscularis mucosae of the guinea-pig oesophagus (Poole *et al*., 2006).

The 10 5-HT$_4$ receptor isoforms that have been identified in human tissues exhibit differential tissue distribution patterns (Medhurst *et al*., 2001; Vilaro *et al*., 2002; Brattelid *et al*., 2004) and the functional significance of these isoforms, which differ (with the exception of 5-HT$_{4(h)}$) in the C terminal tail region is unclear. There is no evidence for an influence of C-terminal splice variant identity on 5-HT$_4$ receptor binding affinity (e.g. Brattelid *et al*., 2004; Krobert *et al*., 2005). However, subtle differences in agonist potency and efficacy, receptor constitutive activity, signal transduction pathways and receptor desensitization properties have been reported for the different splice variants (Claeysen *et al*., 1999; Bender *et al*., 2000; Pindon *et al*., 2002; Mialet *et al*., 2003). For the guinea-pig, although a sequence encoding a single 5-HT$_4$ receptor variant was submitted to Genebank

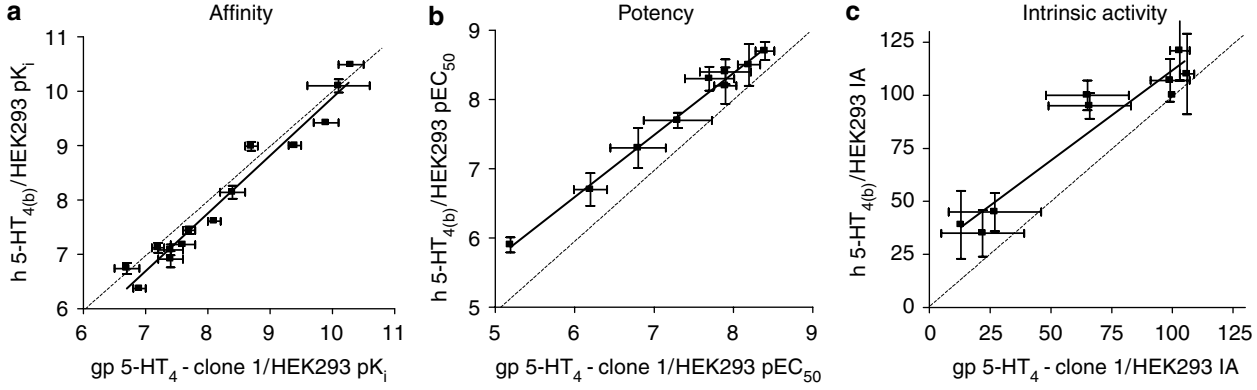

Figure 5 Correlation plots for a range of 5-HT$_4$ receptor agonist and antagonist pK_i, pEC_{50} and IA values at recombinant guinea-pig 5-HT$_4$ and human 5-HT$_{4(b)}$ receptors determined using [^{3}H]GR113808 competition binding assays and cAMP accumulation assays, respectively. The solid lines represent a linear regression through the points, and the dashed lines represent unity. Data represent mean ± s.d. of 3–12 independent experiments.

in 1997, to the best of our knowledge, there has been no publication describing the recombinant receptor. The present study has focused on this variant, which has close amino-acid sequence similarity to the human 5-HT$_{4(b)}$ receptor splice variant and probably represents the guinea-pig orthologue of this receptor. It is hypothesized that in the guinea-pig this is the most abundant form of the receptor. However, a more thorough cloning strategy using primers encoding the specific C-terminal of each receptor variant would be required to definitively rule out the presence of alternative variants.

The pharmacological and functional properties of the guinea-pig recombinant 5-HT$_4$ receptor, in general, were very similar to those of guinea-pig native receptors. As expected, the pK_i values for a range of 5-HT$_4$ receptor selective agonists and antagonists at the recombinant receptor were similar to those at native 5-HT$_4$ receptors in guinea-pig striatal (present study; Uchiyama-Tsuyuki *et al*., 1996) and ileal (Uchiyama-Tsuyuki *et al*., 1996) membrane preparations.

The functional properties of guinea-pig recombinant 5-HT$_4$ receptors were characterized using whole-cell cAMP accumulation studies on two different clones: (i) gp 5-HT$_4$ – clone 2, for which the 5-HT$_4$ receptor density ($\sim$3 pmol mg^{-1} protein) was of a similar magnitude to that reported for several clones that have been used in the characterization of human 5-HT$_4$ receptor splice variants (Bach *et al*., 2001; Pindon *et al*., 2002; Krobert *et al*., 2005) and (ii) gp 5-HT$_4$ – clone 1, for which the 5-HT$_4$ receptor density was at least 10-fold lower ($\sim$0.2 pmol mg^{-1} protein) and proposed to be closer to physiological levels. Thus, although the 5-HT$_4$ receptor density in gp 5-HT$_4$ – clone 1 is 5- to 10-fold higher than that in guinea-pig isolated ileum or proximal colon, it is very similar to that in guinea-pig striatal membranes.

The rank order of 5-HT$_4$ receptor agonist potency, tegaserod $>$5-HT = prucalopride $>$cisapride $>$metoclopramide = mosapride, was similar for the lower receptor density clone and the guinea-pig isolated colon LMMP preparation (present study; Mine *et al*., 1997). Tegaserod was a full agonist (relative to 5-HT) at the guinea-pig recombinant receptor, in contrast to prucalopride, and the benzamide analogues, cisapride and mosapride, which were partial agonists (IA values = 22–76%). Cisapride and mosapride are also 5-HT$_4$ receptor partial agonists (IA values = 37–74%) in isolated tissue preparations from several species including guinea-pig (present study; Bach *et al*., 2001). In the present study, the potency and IA of mosapride (pEC_{50} $<$5.4 and IA $<$37%, respectively) were similar at both the recombinant guinea-pig 5-HT$_4$ receptor and in the guinea-pig LMMP preparation. The present findings suggest that receptor density significantly influences the functional properties of a range of 5-HT$_4$ agonists at the guinea-pig 5-HT$_4$ receptor. There was a significant (5- to 10-fold) increase in agonist potency in the higher receptor density cell line, gp 5-HT$_4$ – clone 2, relative to that in gp 5-HT$_4$ – clone 1, although the rank order of agonist potency was unchanged. In addition, all the agonists tested were full agonists (relative to 5-HT) in gp 5-HT$_4$– clone 2. Therefore, consistent with previous observations at the human 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptors (Pindon *et al*., 2002), the partial agonist cisapride behaved as full agonist in the higher receptor density clone.

Several authors have noted a similar dependence of 5-HT$_4$ agonist potencies and/or IA values on human 5-HT$_4$ receptor density (Bach *et al*., 2001; Krobert *et al*., 2005). For example, a receptor density-dependent increase in agonist efficacy is thought to underlie the different IA values for prucalopride at human 5-HT$_{(4a,\ b,\ c\ and\ i)}$ receptor splice variants (Krobert *et al*., 2005). These observations are consistent with the operational model of agonism (Black and Leff, 1983). Thus, at a higher density of transfected receptors there is amplification of the receptor-induced signals, that is spare receptors (Bruheim *et al*., 2003).

It is well established that tegaserod is a potent, partial agonist at 5-HT$_4$ receptors in rodent isolated tissue preparations (Buchheit *et al*., 1991; Beattie *et al*., 2004). In contrast, tegaserod was a full agonist at the recombinant guinea-pig 5-HT$_4$ receptor, when expressed at either low or high receptor density. Tegaserod is also a potent, full agonist at human 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptor splice variants (Pindon *et al*., 2002). Further investigation is warranted to understand the apparent discrepancies between the isolated tissue and recombinant data. Recently, De Maeyer *et al*. (2006), using the operational model of agonism, demonstrated that the efficacy of tegaserod is in fact similar (equivalent τ-values) to that of 5-HT in porcine atrium and stomach *in vitro* preparations. The potency of tegaserod, however, was lower than expected and the authors attributed this to poor tissue penetration and/or partial precipitation, because of low solubility. Other factors which also may influence the tegaserod response in the recombinant and isolated tissue preparations are differential receptor binding kinetics, receptor reserve, splice variant identity, stimulus-response coupling efficiency, activation of alternate signalling pathways and/or receptor desensitization. However, such factors might be expected to influence the response to other 5-HT$_4$ receptor agonists unless phenomena like agonist-directed trafficking of the stimulus response occurs. To date, there is no evidence that agonist-directed trafficking of 5-HT$_4$ receptors, as has been previously described for the 5-HT$_{2(a)}$ and 5-HT$_{2(c)}$ receptors (Berg *et al*., 1998), occurs. Alternatively, tegaserod may interact with non-5-HT$_4$ receptors in the tissue preparations, thereby influencing the magnitude of 5-HT$_4$ receptor-mediated responses. However, the contribution of 5-HT$_4$ receptors to the tegaserod-evoked contractile responses in digestive tract tissues has been confirmed using 5-HT$_4$ receptor selective antagonists such as piboserod (present study; Beattie *et al*., 2004). Collectively, these data suggest that tegaserod is a full agonist at 5-HT$_4$ recombinant receptors and additional factors underlie its apparent poor efficacy in tissue preparations.

Finally, the present study has demonstrated that the pharmacological and functional properties of the guinea-pig recombinant 5-HT$_4$ receptor are very comparable to those of human recombinant 5-HT$_{4(b)}$ receptor splice variants (Mialet *et al*., 2000; Pindon *et al*., 2002). Given the potential influence of receptor density on the functional properties of human (Bach *et al*., 2001; Bruheim *et al*., 2003; Krobert *et al*., 2005) and/or guinea-pig (present study) 5-HT$_4$ receptors, two clones that expressed the respective

receptors at a similar density ($B_{max} = 0.21 \pm 0.03$ and 0.34 ± 0.03 pmol mg^{-1} protein, respectively) were selected for the comparison in the present study. There was a strong correlation between the agonist potency values for the human and guinea-pig recombinant receptors and very good agreement between the corresponding IA values, with the exception of prucalopride and cisapride, which behaved as full agonists in the human 5-HT$_{4(b)}$ recombinant cell line. A number of investigators have evaluated the pharmacological properties of 5-HT$_4$ receptors in human isolated tissue preparations (Tam *et al*., 1995; Prins *et al*., 2000b; Leclere *et al*., 2005). In spite of the significant limitations associated with use of such tissue preparations there was a reasonable correlation with the guinea-pig recombinant data.

In conclusion, the present study has demonstrated that ligand binding affinities and functional properties at the guinea-pig recombinant 5-HT$_4$ receptor are similar to those at guinea-pig native receptors, in striatal and colonic tissue, respectively. Furthermore, the guinea-pig recombinant 5-HT$_4$ receptor and human 5-HT$_{4(b)}$ receptor splice variant share very similar pharmacological properties. Collectively, these data suggest that pharmacological models using the guinea-pig recombinant 5-HT$_4$ receptor and/or isolated colon preparations represent relevant, and important, tools for the identification and/or characterization of novel 5-HT$_4$ receptor agonists. Such agents may offer improved efficacy for the treatment of disorders of reduced gastrointestinal motility. The pharmacological rationale for the different agonist profiles of tegaserod and the extent to which this may influence the clinical efficacy of tegaserod as a prokinetic agent require elucidation.

Conflict of interest

The authors state no conflict of interest.

References

Alaradi O, Barkin JS (2002). Irritable bowel syndrome: update on pathogenesis and management. *Med Princ Pract* **11**: 2–17.

Bach T, Syversveen T, Kvingedal AM, Krobert KA, Brattelid T, Kaumann AJ *et al.* (2001). 5HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptors have nearly identical pharmacology and are both expressed in human atrium and ventricle. *Naunyn Schmiedebergs Arch Pharmacol* **363**: 146–160.

Barnes NM, Sharp T (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* **38**: 1083–1152.

Barthet G, Gaven F, Framery B, Shinjo K, Nakamura T, Claeysen S *et al.* (2005). Uncoupling and endocytosis of 5-HT$_4$ receptors. Distinct molecular events with different GRK2 requirements. *J Biol Chem* **280**: 27924–27934.

Beattie DT, Smith JA, Marquess D, Vickery RG, Armstrong SR, Pulido-Rios T *et al.* (2004). The 5-HT$_4$ receptor agonist, tegaserod, is a potent 5-HT$_{2B}$ receptor antagonist *in vitro* and *in vivo*. *Br J Pharmacol* **143**: 549–560.

Bender E, Pindon A, van Oers I, Zhang YB, Gommeren W, Verhasselt P *et al.* (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.

Berg KA, Clarke WP, Maayani S, Goldfarb J (1998). Pleiotropic behavior of 5-HT2A and 5-HT2C receptor agonists. *Ann NY Acad Sci* **861**: 104–110.

Black JW, Leff P (1983). Operational models of pharmacological agonism. *Proc R Soc London Ser B* **220**: 141–162.

Brattelid T, Kvingedal AM, Krobert KA, Andressen KW, Bach T, Hystad ME *et al.* (2004). Cloning, pharmacological characterisation and tissue distribution of a novel 5-HT$_4$ receptor splice variant, 5-HT$_{4(i)}$. *Naunyn Schmiedebergs Arch Pharmacol* **369**: 616–628.

Bruheim S, Krobert KA, Andressen KW, Levy FO (2003). Unaltered agonist potency upon inducible 5-HT$_{7(a)}$ but not 5-HT$_{4(b)}$ receptor expression indicates agonist-independent association of 5-HT$_{7(a)}$ receptor and Gs. *Receptor Channel* **9**: 107–116.

Buchheit KH, Gamse R, Pfannkuche HJ (1991). SDZ 205–557, a selective antagonist at 5-HT$_4$ receptors in the isolated guinea pig ileum. *Eur J Pharmacol* **200**: 373–374.

Camilleri M (2004). Advances in pharmacological treatments of IBS. *J Pediatr Gastroenterol Nutr* **39**: S766–S767.

Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108.

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.

Cohen ML, Susemichel AD, Bloomquist W, Robertson DW (1994). 5-HT$_4$ receptors in rat but not guinea pig, rabbit or dog esophageal smooth muscle. *Gen Pharmacol* **25**: 1143–1148.

De Maeyer JH, Prins NH, Schuurkes JA, Lefebvre RA (2006). Differential effects of 5-HT$_4$ receptor agonists at gastric versus cardiac receptors: an operational framework to explain and quantify organ specific behaviour. *J Pharmacol Exp Ther* **317**: 955–964.

Emmanuel AV, Roy AJ, Nicholls TJ, Kamm MA (2002). Prucalopride, a systemic enterokinetic, for the treatment of constipation. *Aliment Pharmacol Ther* **16**: 1347–1356.

Galligan JJ, Pan H, Messori E (2003). Signaling mechanism coupled to 5-HT$_4$ receptor-mediated facilitation of fast synaptic transmission in the guinea-pig ileum myenteric plexus. *Neurogastroenterol Motil* **5**: 523–529.

Gerald C, Adham N, Kao HT, Olsen MA, Laz TM, Schechter LE *et al.* (1995). The 5-HT$_4$ receptor: molecular cloning and pharmacological characterization of two splice variants. *EMBO J* **14**: 2806–2815.

Gershon MD (1999). Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Aliment Pharmacol Ther* **13**: 15–30.

Goldhill J, Porquet MF, Angel I (1997). Post-synaptic 5-HT$_4$ receptor modulation of tachykinergic excitation of rat oesophageal tunica muscularis mucosae. *Eur J Pharmacol* **323**: 229–233.

Grider JR, Foxx-Orenstein AE, Jin JG (1998). 5-HT$_4$ receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. *Gastroenterology* **115**: 370–380.

Grossman CJ, Kilpatrick GJ, Bunce KT (1993). Development of a radioligand binding assay for 5-HT$_4$ receptors in guinea-pig and rat brain. *Br J Pharmacol* **3**: 618–624.

Hegde SS, Eglen RM (1996). Peripheral 5-HT$_4$ receptors. *FASEB J* **10**: 1398–1407.

Hinschberger A, Butt S, Lelong V, Boulouard M, Dumuis A, Dauphin F *et al.* (2003). New benzo[h][1,6]naphthyridine and azepino[3,2-c]quinoline derivatives as selective antagonists of 5-HT$_4$ receptors: binding profile and pharmacological characterization. *J Med Chem* **46**: 138–147.

Hoyer D, Hannon JP, Martin GR (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol Biochem Behav* **71**: 533–554.

Jin JG, Foxx-Orenstein AE, Grider JR (1999). Propulsion in guinea pig colon induced by 5-hydroxytryptamine (HT) via 5-HT$_4$ and 5-HT$_3$ receptors. *J Pharmacol Exp Ther* **288**: 93–97.

Johanson JF (2004). Review article: tegaserod for chronic constipation. *Aliment Pharmacol Ther* **20**: 20–24.

Kajita S, Ito C, Kawamura R, Yasuda S, Isobe Y, Fukushima K (2001). Pharmacological characterization of a novel 5-HT$_4$ receptor agonist, TS-951, *in vitro*. *Pharmacology* **63**: 8–16.

Kenakin T (2002). Drug efficacy at G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **42**: 349–379.

Krobert KA, Brattelid T, Levy FO, Kaumann AJ (2005). Prucalopride is a partial agonist through human and porcine atrial 5-HT$_4$

receptors: comparison with recombinant human 5-HT$_4$ splice variants. *Naunyn Schmiedebergs Arch Pharmacol* **371**: 473–479.
Lacy BE, Yu S (2002). Tegaserod: a new 5-HT$_4$ agonist. *J Clin Gastroenterol* **34**: 27–33.
Leclere PG, Lefebvre RA (2002). Presynaptic modulation of cholinergic neurotransmission in the human proximal stomach. Cloning and characterization of a novel human 5-HT$_4$ receptor variant that lacks the alternatively spliced carboxy terminal exon. *Br J Pharmacol* **135**: 135–142.
Leclere PG, Prins NH, Schuurkes JA, Lefebvre RA (2005). 5-HT$_4$ receptors located on cholinergic nerves in human colon circular muscle. *Neurogastroenterol Motil* **17**: 366–375.
Leung E, Pulido-Rios MT, Bonhaus DW, Pekins LA, Zeitung KD, Hsu SA *et al*. (1996). Comparison of 5-HT$_4$ receptors in guinea-pig colon and rat oesophagus: effects of novel agonists and antagonists. *Naunyn Schmiedebergs Arch Pharmacol* **354**: 145–156.
Liu M, Geddis MS, Wen Y, Setlik W, Gershon MD (2005). Expression and function of 5-HT$_4$ receptors in the mouse enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* **289**: 1148–1163.
Medhurst AD, Lezoualc'h F, Fischmeister R, Middlemiss DN, Sanger GJ (2001). Quantitative mRNA analysis of five C-terminal splice variants of the human 5-HT$_4$ receptor in the central nervous system by TaqMan real time RT-PCR. *Brain Res Mol Brain Res* **90**: 125–134.
Mialet J, Berque-Bestel I, Eftekhari P, Gastineau M, Giner M, Dahmoune Y *et al*. (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 cell lines. *Br J Pharmacol* **129**: 771–781.
Mialet J, Fischmeister R, Lezoualc'h F (2003). Characterization of human 5-HT$_{4(d)}$ receptor desensitization in CHO cells. *Br J Pharmacol* **138**: 445–452.
Mine Y, Yoshikawa T, Oku S, Nagai R, Yoshida N, Hosoki K (1997). Comparison of effect of mosapride citrate and existing 5-HT$_4$ receptor agonists on gastrointestinal motility *in vivo* and *in vitro*. *J Pharmacol Exp Ther* **283**: 1000–1008.
Norum JH, Hart K, Levy FO (2003). Ras-dependent ERK activation by the human G(s)-coupled serotonin receptors 5-HT$_{4(b)}$ and 5-HT$_{7(a)}$. *J Biol Chem* **278**: 3098–3104.
Pauwels PJ (2000). Diverse signaling by 5-hydroxytryptamine (5-HT) receptors. *Biochem Pharmacol* **60**: 1743–1750.
Pindon A, van Hecke G, van Gompel P, Lesage AS, Leysen JE, Jurzak M (2002). Differences in signal transduction of two 5-HT$_4$ receptor splice variants: compound specificity and dual coupling with Gαs- and Gαi/o-proteins. *Mol Pharmacol* **61**: 85–96.
Prins NH, Akkermans LM, Lefebvre RA, Schuurkes JA (2000a). 5-HT$_4$ receptors on cholinergic nerves involved in contractility of canine and human large intestine longitudinal muscle. *Br J Pharmacol* **131**: 927–932.
Prins NH, Shankley NP, Welsh NJ, Briejer MR, Lefebvre RA, Akkermans LM *et al*. (2000b). An improved *in vitro* bioassay for the study of 5-HT$_4$ receptors in the human isolated large intestinal circular muscle. *Br J Pharmacol* **129**: 1601–1608.
Poole DP, Xu B, Koh SL, Hunne B, Coupar IM, Irving HR *et al*. (2006). Identification of neurons that express 5-HT$_4$ receptors in intestine. *Cell Tissue Res* **3**: 413–422.
Reeves DC, Lummis SC (2002). The molecular basis of the structure and function of the 5-HT$_3$ receptor: a model ligand-gated ion channel. *Mol Membrane Biol* **19**: 11–26.
Sakurai-Yamashita Y, Yamashita K, Kanematsu T, Taniyama K (1999). Localization of the 5-HT$_4$ receptor in the human and the guinea pig colon. *Eur J Pharmacol* **383**: 281–285.
Sanger GJ (1996). 5-Hydroxytryptamine and functional bowel disorders. *Neurogastroenterol Motil* **8**: 319–331.
Saxena PR (1995). Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacol Ther* **66**: 339–368.
Tam FS, Hillier K, Bunce KT, Grossman C (1995). Differences in response to 5-HT$_4$ receptor agonists and antagonists of the 5-HT$_4$-like receptor in human colon circular smooth muscle. *Br J Pharmacol* **115**: 172–176.
Uchiyama-Tsuyuki Y, Saitoh M, Muramatsu M (1996). Identification and characterization of the 5-HT$_4$ receptor in the intestinal tract and striatum of the guinea pig. *Life Sci* **59**: 2129–2137.
van den Wyngaert I, Gommeren W, Verhasselt P, Jurzak M, Leysen J, Luyten W *et al*. (1997). Cloning and expression of a human serotonin 5-HT$_4$ receptor cDNA. *J Neurochem* **69**: 1810–1819.
Vilaro MT, Domenech T, Palacios JM, Mengod G (2002). RT-PCR distribution in human brain and periphery of multiple 5-HT$_4$ receptor variants. *Neuropharmacology* **42**: 60–73.
Wardle KA, Sanger GJ (1993). The guinea-pig distal colon – a sensitive preparation for the investigation of 5-HT$_4$ receptor-mediated contractions. *Br J Pharmacol* **4**: 1593–1599.
Yoshikawa T, Yoshida N, Mine Y, Hosoki K (1998). Affinity of mosapride citrate, a new gastroprokinetic agent, for 5-HT$_4$ receptors in guinea pig ileum. *Jpn J Pharmacol* **77**: 53–59.