Contribution of Active and Inactive States of the Human 5-HT<sub>4d</sub> Receptor to the Functional Activities of 5-HT<sub>4</sub>–Receptor Agonists

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Abstract. In the present study, binding affinities of 5-hydroxytryptamine-4 (5-HT<sub>4</sub>) ligands for the human 5-HT<sub>4d</sub> receptor were determined using the agonist [<sup>3</sup>H]5-HT and the selective 5-HT<sub>4</sub> antagonist [<sup>3</sup>H]GR113,808. We also compared the affinity differences between [<sup>3</sup>H]5-HT binding (K<sub>H</sub>) and [<sup>3</sup>H]GR113,808 binding (K<sub>L</sub>) with their activities as 5-HT<sub>4</sub> ligands. Binding studies using [<sup>3</sup>H]5-HT revealed that the human 5-HT<sub>4d</sub> receptor has two binding sites, whereas [<sup>3</sup>H]GR113,808 yielded a single binding site. Additionally, the number of [<sup>3</sup>H]5-HT binding sites decreased in the presence of guanosine-5′-O-(3-thiotriphosphate) (GTP<sub>γ</sub>S), but the number of [<sup>3</sup>H]GR113,808 sites did not change. In competitive binding assays, full agonists such as 5-methoxytryptamine and tegaserod showed 2- to 8-fold higher affinities for [<sup>3</sup>H]5-HT binding (K<sub>H</sub>) than for [<sup>3</sup>H]GR113,808 binding (K<sub>L</sub>) (K<sub>H</sub>&lt;K<sub>L</sub>). Conversely, antagonists showed lower affinities for [<sup>3</sup>H]5-HT binding than for [<sup>3</sup>H]GR113,808 binding (K<sub>H</sub>&gt;K<sub>L</sub>). Finally, partial agonists displayed similar binding affinities for both radioligands (K<sub>H</sub>=K<sub>L</sub>). These findings suggest that the equilibrium between active and inactive states of the human 5-HT<sub>4d</sub> receptor relies on the functional activities of 5-HT<sub>4</sub> ligands, and these states affect the affinities of 5-HT<sub>4</sub> ligands in the competitive binding assay.

Keywords: 5-hydroxytryptamine-4 (5-HT<sub>4</sub>) receptor, agonist, antagonist, serotonin, GR113808

Introduction

Serotonin 5-HT<sub>4</sub> receptors, a subclass of the serotonin (5-hydroxytryptamine, 5-HT)-receptor family, are G-protein–coupled receptors (GPCR) that are positively coupled to adenylyl cyclase (1). The 5-HT<sub>4</sub> receptor was first cloned from rat brain by Gerald et al. who identified two isoforms that differ in the length and sequence of their C-termini (2, 3). cDNAs of 5-HT<sub>4</sub> receptors from mouse, rat, and human were subsequently isolated.

In humans, eight C-terminal splice variants have been cloned (4). The expression and distribution of these splice variants differs among organs and tissues with many of them present in several tissues such as atrium, brain, and GI tract. Notably, the human 5-HT<sub>4d</sub> receptor is reported to only be present in the intestine (5) and the physiological relevance of this receptor in the GI tract may depend on its expression level relative to other splice variants. Therefore it is conceivable that the human 5-HT<sub>4d</sub> receptor could serve as a drug target for the treatment of gastrointestinal disorders such as gastroesophageal reflux disease or irritable bowel syndrome.

The human 5-HT<sub>4d</sub> receptor has a short form of the C-terminus with a truncation of two amino acids after the Leu<sup>359</sup> splice site (5). Functional studies using 5-HT<sub>4</sub>–receptor splice variants has revealed that differences are observed in their signaling properties, that is, coupling to G-proteins, and their functional activity towards chemical tools (4). These findings indicate that investigating the differential function and localization of specific 5-HT<sub>4</sub>–receptor splice variants are important to...
clearly elucidate their pathophysiological role.

Previously, 5-HT₄ agonists have been discovered and developed by using a tritium-labeled selective 5-HT₄ antagonist, [³H]GR113,808, owing to its high affinity, specificity, and reversible property for 5-HT₄ receptors (3). However, differences in Kᵢ values were observed when using agonists versus antagonists as radioligands in competition binding experiments for this receptor. The binding affinities of agonists for the cloned rat 5-HT₄ receptor determined by [³H]GR113,808 were found to be 4- to 20-fold lower than those obtained with the endogenous serotonin agonist, [³H]5-HT (6). Similar observations have been shown in displacement experiments for other GPCRs such as 5-HT₂A, cannabinoid CB1, and dopamine D₂ receptors using both a radio-labeled agonist and an antagonist (7–9). In these observations, agonist binding to GPCRs can be described as a high affinity site that is guanine nucleotide–sensitive and corresponds to a receptor coupled to an empty G-protein, whereas a low affinity site corresponds to a receptor uncoupled with G-protein and close to the ground state (R). The ratio of the affinities for these two states represents how well an agonist stabilizes the ternary complex and correlates with agonist efficacy (10).

This hypothesis has been applied to 5-HT₄ receptors and there are reports suggesting that the ratio of the low affinity site to the high affinity site can be attributed to the active and inactive states of these receptors (11, 12). Moreover, there are discrepancies between the binding affinities of agonists determined by competition binding assay with [³H]GR113,808 and their functional potencies for the 5-HT₄ receptor. Bender et al. reported that Kᵢ values of 5-HT₄ agonists determined by [³H]GR113,808 were more than 10 times higher than those of EC₅₀ values in human 5-HT₄a and 5-HT₄b receptors and that the ratio of the Kᵢ value for the low affinity site to that for the high affinity site could be correlated to the functional efficacy at 5-HT receptors (11). These observations indicate that the intermediate transition states (active and inactive) of receptors cause a different binding mode between agonist and antagonist and affect the functional activities of agonists. The ratio of the Kᵢ value for the low affinity site to that for the high affinity site between agonist binding and antagonist binding is possibly attributed to the existence of an active state or inactive state of G-protein–coupled receptors and conformational induction model (13). It also has been reported that the human 5-HT₄a receptor can generate a spontaneously active receptor site, indicating that the receptor exists in an active (coupled with G-protein) state (11, 12, 14).

However, there has been no pharmacological study of 5-HT₄ ligands to characterize and compare their binding affinity and function on the human 5-HT₄ receptor. Also, it remains to be determined if there is a correlation between the ratio of the Kᵢ value for the low affinity and high affinity sites of 5-HT₄ ligands determined by competitive binding using an agonist and antagonist as radioligands and their functional activities at this receptor. Furthermore, it is still unclear how the high affinity state of the receptor contributes to receptor coupling with the G-protein. Therefore, this study aimed to determine the pharmacological profile of the 5-HT₄ ligands on the human 5-HT₄ receptor using the agonist [³H]5-HT and selective 5-HT₄ antagonist [³H]GR113,808 as radioligands. We also sought to compare their binding and functional activity by using the increase in cAMP induced by the ligands. Finally, we investigated the effect of the non-hydrolyzable GTP analog GTPγS on 5-HT or GR113,808 binding to the human 5-HT₄ receptor to assess whether the human 5-HT₄ receptor is coupled with G-protein.

Our results indicate that 5-HT binding at the human 5-HT₄ receptor showed two affinity sites, while GR113,808 binding yielded a single site. Moreover the number of 5-HT binding sites significantly decreased in the presence of GTPγS but that of GR113,808 did not, indicating that the human 5-HT₄ receptor exists both in an active (coupled with G-protein) and an inactive state (uncoupled with G-protein). We also demonstrated that full agonists stabilize the active state of the human 5-HT₄ receptor, whereas an antagonist does not affect the G-protein–coupling to the receptor. These findings suggest that the equilibrium between the active and inactive state of the human 5-HT₄ receptor relies on the functional activity of 5-HT₄ ligands, and these states affect the affinities of 5-HT₄ ligands in the competitive binding assay.

Materials and Methods

Materials

GR113,808 [(1-(2-((methylsulphonyl)amino)ethyl)-4-piperidinyl)methyl-1-methyl-1H-indole-3-carboxylate], SB204,070 (1-butyl-4-piperidinylmethyl-8-amino-7-chloro-1-4-benzoioxan-5-carboxylate), GR125,487 [5-fluoro-2-methoxy-[1-[2[(methylsulfonyl)amino]ethyl]-4-piperidinyl]-1H-indole-3-methylcarboxylate sulfamate], S67,333 [1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-butyl-4-piperidinyl]-1-propanone, and RS67,506 [1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1-methylsulphonylamino]ethyl-4-piperidinyl]-1-propanone] were from Tocris Bioscience (Ellisville, MO, USA). [³H]GR113,808 (specific activity of 81.0 Ci/mmol), [³H]5-HT (specific activity of 80.0 Ci
chloro[4-amino-5-chloro-N-[1-[3-(4-fluorophenoxo)propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide], tegaserod [2-((5-methoxy-1H-indol-3-yl)methylene)-N-pentylhydrazinecarboximidamide], and mosapride [2-((5-methoxy-2-ethoxy-N-[4-[(4-fluorophenyl)methyl]-2-morpholinyl]methyl)-benzamide] were synthesized at Pfizer

4d

expressing the human 5-HT receptor. For the agonist binding assay, membrane homogenate (300 μg) and [3H]5-HT (final concentration: 3.0 – 70 nM) were incubated for 60 min at 25°C in the absence or presence of 50 μM GR113,808 (final concentration) to determine total or non-specific binding, respectively. For the antagonist binding assay, membrane homogenate (10 μg) and [3H]GR113,808 (final concentration: 0.02 – 2.0 nM) were incubated for 60 min at room temperature in the absence or presence of 1 μM GR113,808 (final concentration) for total or non-specific binding, respectively. All incubations were terminated by rapid vacuum filtration over 1% polyethyleneimine (PEI; Wako, Osaka)-soaked glass fiber filter paper followed by two washes with 50 mM HEPES (pH 7.4) using a Brandel cell harvester (Brandel, Gaithersburg, MD, USA) and a Skatron harvester (Perkin-Elmer, Waltham, MA, USA) for the agonist or antagonist binding studies, respectively. Receptor-bound radioactivity was quantified by liquid scintillation counting using a Betaplate counter (PerkinElmer).

Membrane preparation

HEK293 cells expressing the human 5-HT4d receptor were harvested and homogenized with 50 mM HEPES (pH 7.4) supplemented with protease inhibitor cocktail (Sigma-Aldrich). The cell suspension was homogenized and then centrifuged at 40,000 × g at 4°C. The pellet was resuspended into 50 mM HEPES buffer (pH 7.4), homogenized a second time, and centrifuged as described above. The final pellet was resuspended and homogenized in 50 mM HEPES buffer (pH 7.4). Each membrane fraction was stored at -80°C until use. Protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Radioligand binding assay

Saturation experiments for [3H]5-HT and [3H]GR113,808 were conducted to establish the equilibrium binding parameter for the human 5-HT4d receptor. For the agonist binding assay, membrane homogenate (300 μg) and [3H]5-HT (final concentration: 3.0 – 70 nM) were incubated for 60 min at 25°C in the absence or presence of 50 μM GR113,808 (final concentration) to determine total or non-specific binding, respectively. For the antagonist binding assay, membrane homogenate (10 μg) and [3H]GR113,808 (final concentration: 0.02 – 2.0 nM) were incubated for 60 min at room temperature in the absence or presence of 1 μM GR113,808 (final concentration) for total or non-specific binding, respectively. All incubations were terminated by rapid vacuum filtration over 1% polyethyleneimine (PEI; Wako, Osaka)-soaked glass fiber filter paper followed by two washes with 50 mM HEPES (pH 7.4) using a Brandel cell harvester (Brandel, Gaithersburg, MD, USA) and a Skatron harvester (Perkin-Elmer, Waltham, MA, USA) for the agonist or antagonist binding studies, respectively. Receptor-bound radioactivity was quantified by liquid scintillation counting using a Betaplate counter (PerkinElmer).

Compartment experiments for agonist and antagonist binding were conducted to determine the affinities of synthetic 5-HT4 ligands for the human 5-HT4d receptor. For the agonist binding assay, membrane homogenate (300 μg) and [3H]5-HT (final concentration: 5.0 – 9.0 nM) were incubated for 60 min at 25°C with serially diluted synthetic 5-HT4 ligands. All incubations were terminated by rapid vacuum filtration over PEI-soaked glass fiber filter paper using the Brandel cell harvester followed by two washes with 50 mM HEPES (pH 7.4). Receptor-bound radioactivity was quantified by liquid scintillation counting using the Betaplate counter. Non-specific binding was determined by the addition of a final concentration of 50 μM GR113,808. For the antagonist binding assay, membrane homogenate (10 μg), SPA beads, and [3H]GR113,808 (final 0.2 nM) were incubated for 60 min at 25°C with serially diluted synthetic 5-HT4 ligands. All incubations were terminated by centrifuging at 1,000 rpm. Receptor-bound radioactivity was quantified by counting with a Micro-Beta plate counter (PerkinElmer). Non-specific binding was determined by the addition of a final concentration of 1 μM GR113,808.

Experiments with GTPγS for agonist and antagonist binding were conducted to assess whether the human 5-HT4d receptor labeled with [3H]5-HT and that labeled with [3H]GR113,808 are coupled with G-protein. Both [3H]5-HT (10 nM) and [3H]GR113,808 (0.2 nM) were used as radioligands in the absence or presence of GTPγS (0.1 – 1,000 μM).

Cyclic AMP (cAMP) assay

The human 5-HT4d receptor transfected HEK293 cells (h5-HT4d/HEK293) were used to conduct the cAMP
For measurement of intracellular cAMP production, cAMP concentration in the mixture of each well was measured by a HTRF-cAMP dynamic kit (CisBio; Bagnols/Cèze Cedex, France). h5-HT_4d/HEK293 cells were grown to approximately 60%–80% confluence. The medium was changed to DMEM containing 10% dialyzed FCS, and then the cells were incubated overnight to remove endogenous 5-HT. The cells were harvested with PBS/1 mM EDTA, centrifuged, and washed with PBS. The cell pellet was resuspended in DMEM supplemented with 20 mM HEPES, 10 µM pargyline, and 1 mM 3-isobutyl-1-methylxanthine (IBMX) at the concentration of 1.6 × 10^5 cells/ml and incubated for 15 min at room temperature. The reaction was initiated by addition of the cells onto plates (2 × 10^3 cells/well) containing serially diluted test compounds. After incubation for 15 min at room temperature, 1% Triton X-100 (Wako) was added to stop the reaction. After lysis for 30 min at room temperature, cAMP-XL665 conjugate was added to the lysate followed by anti-cAMP–cryptate conjugate. After further incubation for 60 min at room temperature, measurements were made on a 1420 ARVOsx multilabel counter (PerkinElmer). To determine the maximal elevation of cAMP, 1 μM of 5-HT was used. Data analysis was made based on the ratio of fluorescence intensity of each well at 620 and 665 nm. Enhancement of cAMP production elicited by each compound was normalized to the amount of cAMP elevated by 1 μM 5-HT. Curve fitting was performed with GraphPad Prism version 3.02 software (GraphPad, San Diego, CA, USA).

**Results**

**Characterization of \[^3H\]5-HT and \[^3H\]GR113,808 binding to the human 5-HT_4d receptor**

The K_D and B_max values of \[^3H\]5-HT and \[^3H\]GR113,808 for the human 5-HT_4d receptor were obtained by Scatchard plot analyses of data derived from saturation experiments. The Scatchard plot of \[^3H\]5-HT clearly showed two binding sites, one with a high affinity and one with a low affinity, at the human 5-HT_4d receptor in the dose range of 3.0 – 70 nM (Fig. 1A). On the other hand, \[^3H\]GR113,808 binding appeared to yield a single site in the dose range of 0.02 – 2.0 nM (Fig. 1B). The K_D and B_max values of \[^3H\]5-HT and \[^3H\]GR113,808 for the human 5-HT_4d receptor are shown in Table 1. Scatchard analysis using

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>K_D (nM)</th>
<th>B_max (pmol/mg protein)</th>
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<tbody>
<tr>
<td>[^3H]5-HT (n = 3)</td>
<td></td>
<td></td>
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<tr>
<td>High affinity site</td>
<td>8.5 ± 1.1</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>Low affinity site</td>
<td>91 ± 48</td>
<td>0.92 ± 0.46</td>
</tr>
<tr>
<td>[^3H]GR113,808 (n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single site</td>
<td>0.11 ± 0.03</td>
<td>1.3 ± 0.1</td>
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</tbody>
</table>

Calculation was performed by using non-linear regression with Prism ver. 3.02 software. Values are each the mean ± S.D. from at least three independent experiments performed in duplicate or triplicate.

![Fig. 1. Saturation curve and Scatchard plot of \[^3H\]5-HT and \[^3H\]GR113,808 binding to human 5-HT_4d receptors. Cell membrane of the human 5-HT_4d receptor was incubated with 8 – 10 concentrations of either \[^3H\]5-HT (3.0 – 70 nM) or \[^3H\]GR113,808 (0.02 – 2.0 nM) for 60 min at 25°C. Non-specific binding was defined using 50 μM (for \[^3H\]5-HT binding) or 1 μM (for \[^3H\]GR113,808 binding) unlabeled GR113,808 in the assay. Results are from one representative experiment of three or four experiments. Graphs were prepared with the GraphPad Prism ver. 3.02. software.](image-url)
[1H]5-HT as the radioligand showed two affinity sites with 

\( K_D \) values of 8.5 nM for the high affinity site and 91 nM for the low affinity site \((n = 3)\). The maximal binding, \( B_{max} \), was 0.19 pmol/mg protein at the high affinity site and 0.92 pmol/mg protein at the low affinity site \((n = 3)\). By comparison, Scatchard analysis for [1H]GR113,808 demonstrated a single affinity site with a \( K_D \) value of 0.11 nM and a \( B_{max} \) value of 1.3 pmol/mg protein for the human 5-HT\(_{4d}\) receptor \((n = 4)\).

**Effect of GTP\(_{\gamma}\)S on [1H]5-HT and [1H]GR113,808 binding to the human 5-HT\(_{4d}\) receptor**

To assess whether the human 5-HT\(_{4d}\) receptor bound with either [1H]5-HT or [1H]GR113,808 is coupled to G-protein, the effect of GTP\(_{\gamma}\)S, a non-hydrolyzable GTP analog, on the binding of both radiolabeled ligands was determined. As shown in Fig. 2, the specific binding of [1H]5-HT was significantly decreased in a dose-dependent manner by GTP\(_{\gamma}\)S, and the maximal inhibition was 77% at 1 mM GTP\(_{\gamma}\)S. On the other hand, GTP\(_{\gamma}\)S at concentrations up to 1 mM did not affect the specific binding of [1H]GR113,808 to the human 5-HT\(_{4d}\) receptor.

**Concentration–response curves of synthetic 5-HT\(_{4}\) ligands for cAMP production at the human 5-HT\(_{4d}\) receptor**

Next, we investigated the functional activities of synthetic 5-HT\(_{4}\) ligands at the human 5-HT\(_{4d}\) receptor. Figure 3 shows the concentration response curves of the radioligands for cAMP production at the human 5-HT\(_{4d}\) receptor. The cells were incubated for 15 min with synthetic 5-HT\(_{4}\) ligands, and cyclic AMP production was then quantified by the HTRF kit. The Y axis is expressed as the percentage of 5-HT maximal response at 1000 nM. Full agonists (A): 5-HT, 5-MeOT, tegaserod, and cisapride; partial agonists (B): Mosapride, RS67,333 and RS67,506; antagonists (C): GR113,808, SB204,070, and GR125,487. EC\(_{50}\) and E\(_{max}\) values (% of 5-HT) of radioligands were derived from curve fitting. Graphs were prepared with the GraphPad Prism ver. 3.02 software. Results are from one representative experiment in three or four experiments.
These full agonists showed various EC₅₀ values in the range of 7 – 167 nM (Table 2). In comparison, Mosapride, RS67,333, and RS67,506 showed partial agonistic activities (Fig. 3B) with E₅₀ values of 52% (EC₅₀: 1,013 nM), 55% (EC₅₀: 2.7 nM), and 54% (EC₅₀: 5.0 nM), respectively. GR113,808, SB204,070, and GR125,487, however, did not stimulate cAMP production (Fig. 3C) with E₅₀ values of 17% or less.

Kᵢ values of synthetic 5-HT₄ ligands, full agonists, partial agonists, and antagonists determined using [³H]5-HT and [³H]GR113,808 as radioligands

We investigated the affinities of 5-HT₄ ligands to the human 5-HT₄ receptor using either [³H]5-HT or [³H]GR113,808 as radioligands (Table 3). Kᵢ represents a Ki value determined using [³H]5-HT as a radioligand and Kᵢ represents a Ki value determined using [³H]GR113,808 as a radioligand. 5-HT showed a Ki value (Kᵢ) of 21 nM in [³H]5-HT binding to the human 5-HT₄ receptor, whereas 5-HT has a Ki value (Kᵢ) of 171 nM in [³H]GR113,808 binding. The Kᵢ/Kᵢ ratio (ratio of the affinity for [³H]GR113,808 binding to that of [³H]5-HT binding) was 8.1. Other full agonists that we tested demonstrated KL/KH ratios in the range of 1.7 – 6.3. By contrast, the antagonists GR113,808, SB204,070, and GR125,487 clearly had lower affinities in [³H]5-HT binding (Kᵢ) than in [³H]GR113,808 binding (Kᵢ), yielding Kᵢ/Kᵢ ratios of 0.10 – 0.15. Finally, partial agonists displayed equivalent binding affinities for both [³H]5-HT binding and [³H]GR113,808 binding as demonstrated by their Kᵢ/Kᵢ ratios of 0.9 – 1.2.

### Table 2. Agonistic activities of synthetic 5-HT₄ ligands for cAMP production in HEK293 cells expressing the human 5-HT₄ receptor

<table>
<thead>
<tr>
<th>Ligands</th>
<th>EC₅₀ (nM)</th>
<th>E₅₀ (% of 5-HT maximal response)</th>
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<tbody>
<tr>
<td>Full agonist</td>
<td></td>
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<tr>
<td>5-HT</td>
<td>19 ± 12</td>
<td>100</td>
</tr>
<tr>
<td>5-MeOT</td>
<td>17 ± 11</td>
<td>106 ± 12</td>
</tr>
<tr>
<td>Tegaserod</td>
<td>7.0 ± 1.7</td>
<td>110 ± 7.5</td>
</tr>
<tr>
<td>Cisapride</td>
<td>167 ± 116</td>
<td>98 ± 7.2</td>
</tr>
<tr>
<td>Partial agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS67,506</td>
<td>5.0 ± 2.6</td>
<td>54 ± 6.8</td>
</tr>
<tr>
<td>RS67,333</td>
<td>2.7 ± 0.6</td>
<td>55 ± 5.3</td>
</tr>
<tr>
<td>Mosapride</td>
<td>1,013 ± 268</td>
<td>52 ± 6.0</td>
</tr>
<tr>
<td>Antagonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR113,808</td>
<td>N.D.</td>
<td>−0.7 ± 1.2</td>
</tr>
<tr>
<td>SB204,070</td>
<td>N.D.</td>
<td>17 ± 8.1</td>
</tr>
<tr>
<td>GR125,487</td>
<td>N.D.</td>
<td>−1.3 ± 1.5</td>
</tr>
</tbody>
</table>

Calculation was performed by using non-linear regression with Prism ver. 3.02 software. Values are each the mean ± S.D. from at least three independent experiments performed in duplicate or triplicate. N.D.: Not determined.

### Table 3. Binding affinities of agonists and antagonists for the human 5-HT₄ receptor determined using [³H]5-HT (Kᵢ) and [³H]GR113,808 (Kᵢ)

<table>
<thead>
<tr>
<th>Ligands</th>
<th>[³H]5-HT (Kᵢ)</th>
<th>[³H]GR113,808 (Kᵢ)</th>
<th>Kᵢ/Kᵢ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full agonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>21 ± 0.8</td>
<td>171 ± 48</td>
<td>8.1</td>
</tr>
<tr>
<td>5-MeOT</td>
<td>107 ± 4.6</td>
<td>674 ± 45</td>
<td>6.3</td>
</tr>
<tr>
<td>Tegaserod</td>
<td>9.5 ± 3.9</td>
<td>25 ± 7</td>
<td>2.6</td>
</tr>
<tr>
<td>Cisapride</td>
<td>81 ± 19</td>
<td>140 ± 15</td>
<td>1.7</td>
</tr>
<tr>
<td>Partial agonist</td>
<td></td>
<td></td>
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<tr>
<td>RS67,506</td>
<td>4.5 ± 0.1</td>
<td>5.2 ± 1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>RS67,333</td>
<td>4.3 ± 0.4</td>
<td>5.3 ± 1.1</td>
<td>1.2</td>
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<tr>
<td>Mosapride</td>
<td>375 ± 197</td>
<td>335 ± 115</td>
<td>0.9</td>
</tr>
<tr>
<td>Antagonist</td>
<td></td>
<td></td>
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<tr>
<td>GR113,808</td>
<td>0.91 ± 0.39</td>
<td>0.14 ± 0.06</td>
<td>0.15</td>
</tr>
<tr>
<td>SB204,070</td>
<td>0.76 ± 0.45</td>
<td>0.083 ± 0.027</td>
<td>0.11</td>
</tr>
<tr>
<td>GR125,487</td>
<td>1.1 ± 0.5</td>
<td>0.12 ± 0.07</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Calculation was performed by using non-linear regression with Prism ver. 3.02 software. Values are each the mean ± S.D. from at least three independent experiments performed in duplicate or triplicate.
Discussion

In this study we determined the binding affinities of several human 5-HT₄ receptor agonists and antagonists using [³H]5-HT and [³H]GR113,808. We also examined the relationship between these affinities (Kᵢ/Kᵢ) and how that ratio reflected the functional efficacy of these 5-HT₄ ligands. Saturation binding of the receptor performed with [³H]GR113,808 showed a single, high affinity binding site by Scatchard analysis, which is consistent with previous reports using transfected cells expressing the human 5-HT₄ receptor (5, 15). By comparison, Scatchard analysis of [³H]5-HT binding exhibited two sites with distinct affinities (one high affinity site and one low affinity site) in our study. Other reported studies have also suggested the existence of two different affinity sites on 5-HT₄ receptors (6). Shallow competition binding curves and partial GTP/γS sensitivity indicated the existence of two different affinity sites. Moreover, Scatchard analysis of data derived from [³H]5-HT saturation binding curves for the human 5-HT₄ and 5-HT₄δ receptors also showed distinct high and low affinity sites when high concentrations of [³H]5-HT but not when low nanomolar concentrations of [³H]5-HT were used (11, 12). These results indicated that high concentrations of [³H]5-HT were needed in the binding assay to reveal the two binding sites by Scatchard analysis. A structural-based study using purified 5-HT₄ receptors has proposed that the conformational changes involved in both receptor activation and inactivation affect the geometrical features of their second extracellular loop. Specifically, these changes were observed upon agonist binding, whereas antagonist binding did not induce similar changes in the conformation of the receptor (16). Thus, based on these findings that the natural agonist [³H]5-HT can label the active state (R* state and R*G state) of 5-HT₄ receptors with high affinity, it is likely that agonists may stabilize coupling of the receptor to the G-protein. Antagonists, on the other hand, label all receptor conformations (including active and inactive states) without affecting the efficiency of G-protein–coupling to 5-HT₄ receptors.

Comparison of B_max values using [³H]GR113,808 and [³H]5-HT allowed us to estimate the equilibrium constant (J) between the active (R*) and the inactive (R) states of the human 5-HT₄ receptor in the HEK293 cells used in our experiment. J = [R]/[R*] and can be estimated by (R_total − R*)/R* (12). In addition, it has been suggested that the constant J is a receptor structural characteristic, which is independent of receptor density (16). Total receptor binding sites (R_total) corresponds to B_max obtained with [³H]GR113,808, while R* corresponds to B_max obtained for the high affinity site with [³H]5-HT. For our results, R_total is 1.3 pmol/mg protein and R* is 0.19 pmol/mg protein. Consequently, the equilibrium constant J is equal to (1.3 − 0.19)/0.19 = 5.8, corresponding to 14.6% of the total 5-HT₄ receptors in the active state.

The non-hydrolyzable GTP analog, GTPγS, inhibits the association of G-protein with the active state of the receptor after one cycle of receptor-induced guanine nucleotide exchange because the G-protein cannot regenerate the GDP-ligated conformation. Under incubation of the receptor with this GTP analog, the equilibrium is shifted to the receptor in the inactive state (8). In the present study, [³H]5-HT binding sites were significantly decreased to 33% in the presence of GTPγS. In this experiment, [³H]5-HT at the concentration of 10 nM was used. As shown in Fig. 1A, a specific binding of 10 nM [³H]5-HT is approximately 0.1 pmol/mg protein. Therefore, 2.5% of the total amount of receptor is estimated to be coupled with G-protein because 33% of the specific binding of [³H]5-HT (0.1 pmol/mg protein) in the presence of GTPγS is 0.033 pmol/mg protein that corresponds to 2.5% of the total amount of the receptor (1.3 pmol/mg protein). On the other hand, specific binding of [³H]GR113,808 did not change even in the presence of up to 1 mM GTPγS, indicating that antagonist binding does not affect the efficiency of G-protein–coupling to the receptor. This result shows that the active state of the receptor was detected at a low concentration of [³H]5-HT. These results can be explained using the two-state model of the receptor in which G-protein–coupled receptors are in equilibrium between the active and the inactive state in the absence of agonists (17). Agonists have high affinity for the active state and displace the equilibrium towards the active state from the inactive state, whereas antagonists have equal affinity for both states and do not displace the equilibrium. As mentioned earlier, antagonists will label all receptors including the active and inactive states without affecting the efficiency of G-protein–coupling in the human 5-HT₄ receptor. Hence, it is most likely that the difference in the equilibrium of receptors between the active and the inactive states may lead to the observed differences in Kᵢ values of 5-HT₄ ligands determined by using a [³H]agonist and a [³H]antagonist as receptor probes.

The 5-HT₄ ligands used in this in vitro study were categorized into three functional groups: full agonists, partial agonists, and antagonists. There are some discrepancies in the functional activities of the 5-HT₄ agonists between our results and those described in other reports. Cisapride has been reported as a 5-HT₄ partial agonist at the human 5-HT₄ receptors,

5-HT₄ receptors.

[50x292]changes in the conformation of the receptor (16). Thus, these changes were observed upon agonist binding, tures of their second extracellular loop. Specifically, activation and inactivation affect the geometrical fea-

[50x330]conformational changes involved in both receptor

[50x455]human 5-HT

[50x617]γ

[50x580]4d

[50x542]affinity site and one low affinity site) in our study. Other

[50x80](16). Total receptor binding sites (R

[50x92]characteristic, which is i ndependent of receptor density

[50x117]partial agonists, and antagonists. There are some
discrepancies in the functional activities of the 5-HT₄
agonists between our results and those described in other
reports. Cisapride has been reported as a 5-HT₄ partial
agonist at the human 5-HT₄ receptors,
18). In our experimental system, however, cisapride exhibited properties of a full agonist at the human 5-HT$_{4d}$ receptor. Functional characterization of 5-HT$_4$ agonists may depend on the type of 5-HT$_4$ splice variants utilized and on receptor density in the membrane for each experimental setup. For example, cisapride has been shown to exert full agonism at the human 5-HT$_{4a}$ and 5-HT$_{4b}$ receptor (12). However, there are other reports describing cisapride as a partial agonist at low receptor expression levels, but as a full agonist at high receptor levels of 5-HT$_4$-receptor splice variants (19, 20). These authors suggested that a high level of basal adenyl cyclase activity could affect the functional efficacy of 5-HT$_4$ agonists. HEK293 cells expressing the human 5-HT$_{4d}$ receptor used in this study did not show the basal adenyl cyclase activity (data not shown). It remains to be elucidated why cisapride exhibited full agonism in the cAMP assay at the human 5-HT$_{4d}$ receptor with no basal adenyl cyclase activity in our study. Similarly, the functional characteristics of GR125,487 may also depend on the type of 5-HT$_4$ splice variants and/or receptor density expressed on the cells used in the experiments. Claeyssen et al. reported that GR125,487 functioned as an inverse agonist for human or mouse 5-HT$_{4a}$ receptors (21). They compared the functional characteristics of inverse agonists for the 5-HT$_4$ receptor using wild type human 5-HT$_{4a}$ receptor and a constitutively active mutant having a different receptor density. Based on their data, they concluded that efficacy of the inverse agonists was dependent on receptor structure but not on receptor density. On the other hand, Brattelid et al. showed that GR125,487 acted as an inverse agonist at the 5-HT$_{4i}$ receptor, but acted as a neutral antagonist at the 5-HT$_{4i}$ receptor (20). They attributed the function of GR125,487 as a neutral antagonist due to the density of 5-HT$_{4i}$ receptors in the membrane used in their study. Furthermore, similar differences in the functional characteristics other 5-HT$_4$ ligands have been observed; ML10375 is an inverse agonist on the human 5-HT$_{4a}$ receptor, but a neutral antagonist on the human 5-HT$_{4d}$ receptor (15, 22). These reported differences in the functional characterization of 5-HT$_4$ ligands at 5-HT$_4$ receptors may relate to the observations and interpretations of our present study.

In the competitive binding assays, full agonists such as 5-MeOT and tegaserod showed 1.7- to 8.1-fold higher affinities for [H]5-HT binding than for [H]GR113,808 binding (K$_{il}$<K$_{il}$). In contrast, antagonists exhibited 6.5- to 10-fold lower affinities for [H]5-HT binding than for [H]GR113,808 (K$_{il}$>K$_{il}$) such that these K$_{il}$/K$_{il}$ were close to approximately 0.1. The partial agonists displayed similar binding affinities for the two radioligands (K$_{il}$ = K$_{in}$). Consequently, our study confirmed the distinction of K$_i$ values for a full agonist versus an antagonist in competing for binding with [H]agonist or [H]antagonist, which is in close accordance with the report by Fitzgerald et al. (23). Their work using human 5-HT$_{2a}$ and 5-HT$_{2c}$ receptors concluded that K$_{il}$/K$_{il}$>1 is indicative of an agonist; K$_{il}$/K$_{il}$ = 1, a partial agonist; and K$_{il}$/K$_{il}$<1, an antagonist. Furthermore, they attributed the differences observed in their results compared to those in other reports (e.g., K$_{il}$/K$_{il}$>1 is an agonist, K$_{il}$/K$_{il}$<1 is an inverse agonist, and K$_{il}$/K$_{il}$ = 1 is an antagonist) to the properties of the in vitro model systems used in the specific experiments (23 – 25). This hypothesis similarly may explain why our conclusions differ from those in the other reports (e.g., K$_{il}$/K$_{il}$>1 is an agonist, K$_{il}$/K$_{il}$<1 is an inverse agonist, and K$_{il}$/K$_{il}$ = 1 is an antagonist). Our results suggest that the relative efficacy of each 5-HT$_4$ ligand in human 5-HT$_4$ receptors is predictable from K$_i$ differences (K$_{il}$/K$_{il}$; [H]5-HT to [H]GR113,808), which is in accordance with several previous reports examining G-protein-coupled receptors (8, 10, 24 – 26). However further investigation will be required to more clearly elucidate the details of whether receptor structure or receptor density affects the functional characteristics of 5-HT$_4$ ligands.

In conclusion, we have defined the relationship between the K$_i$ ratio (K$_{il}$/K$_{il}$) and the functional efficacy of several 5-HT$_4$ ligands at the human 5-HT$_{4d}$ receptor. Our study indicates that full agonists stabilize the active state of the human 5-HT$_{4d}$ receptor, whereas antagonists do not affect the G-protein-coupling to the receptor. Taken together, these findings suggest that the equilibrium between the active state and the inactive state of the human 5-HT$_{4d}$ receptor depends on the specific functional activity of 5-HT$_4$ ligands and that these states affect the affinity of 5-HT$_4$ ligands in the competitive binding assay. Therefore, efficacy of 5-HT$_4$ ligands should be predicted from comparative assessments of their relative binding affinities for the active versus inactive states of the human 5-HT$_{4d}$ receptor.

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**References**


logical characterization of two splice variants. EMBO J. 1995;14:2806–2815.
19 Bach T, Syversveen T, Kvingedal AM, Krobert KA, Brattelid T, Kaumann AJ, et al. 5-HT4(α) and 5-HT4(β) receptors have nearly identical pharmacology and are both expressed in human atrium and ventricle. Naunyn Schmiedebergs Arch Pharmacol. 2001;363:146–160.