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In vitro and in vivo pharmacological characterization of PF-01354082, a novel partial agonist selective for the 5-HT_4 receptor

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ABSTRACT

The pharmacological profile of PF-01354082, a selective 5-HT_4 receptor partial agonist, was investigated. PF-01354082 displayed high affinity for human 5-HT_{4d} and dog 5-HT_{4h} receptors in binding studies, having Ki values of 2.0 nM and 4.2 nM, respectively. By contrast, PF-01354082 did not show significant affinity for several other 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{3A}, and 5-HT_7) or the dopamine D_{2long} receptor. Functional assays using either cells expressing human recombinant 5-HT_{4d} receptors or rat tunica muscularis mucosae demonstrated that PF-01354082 exhibited partial agonist activity at the 5-HT_4 receptor. The effects of PF-01354082 on in vitro receptor binding, ion channel activity, and sites of uptake were further investigated. PF-01354082 did not show biologically relevant binding activity at concentrations up to 10 μM except for binding to the 5-HT_{4e} receptor. Furthermore, PF-01354082 decreased I_{HERG} current by only 11% at a concentration of 300 μM, indicating that the compound had greater than 150,000-fold selectivity for the human 5-HT_{4d} receptor over hERG channels. An in vivo study using a gastric motility model in conscious dogs demonstrated that oral administration of PF-01354082 resulted in marked and sustained stimulation of gastric motility in a dose-dependent manner. These results indicate that PF-01354082 is an orally active, highly selective, partial agonist of the human 5-HT_4 receptor that is expected to exert a favorable effect on gastrointestinal motor disorders with reduced adverse effects mediated by other related receptors.

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1. Introduction

The serotonin (5-hydroxytryptamine, 5-HT) type-4 (5-HT_4) receptor was originally discovered by Dumuis et al. (1988). Subsequently, 5-HT_4 receptor-mediated functional responses were demonstrated using gastrointestinal tissues, such as contraction of the guinea pig ileum longitudinal muscle myenteric plexus (Craig and Clarke, 1990) and relaxation of rat esophageal tunica muscularis mucosae (Baxter et al., 1991). Recently, increasing evidence has confirmed that 5-HT_4 receptors play a crucial role in diverse gastrointestinal functions in humans. For example, the 5-HT_4 receptor agonist, cisapride, has been shown to increase lower esophageal sphincter tone in humans (Pehlivanov et al., 2002), increase esophageal peristalsis (Wienbeck and Li, 1989), and promote gastric emptying (Maddern et al., 1991). Thus, cisapride was demonstrated to be effective in patients with gastroparesis and gastro-esophageal reflux disease. Mosapride, another 5-HT_4 receptor agonist, has shown significant effects to decrease acid reflux and increase esophageal motor function in patients with gastro-esophageal reflux disease (Ruth et al., 1998, 2003), while tegaserod, a 5-HT_4 receptor partial agonist, has exhibited efficacy against esophageal acid exposure in patients with gastro-esophageal reflux disease (Kahrilas et al., 2000) where it was shown to significantly decreased the frequency of occurrence of heart burn, acid reflux, regurgitation, and distress from regurgitation (Rodoriguez-Stanley et al., 2006). Tegaserod is also reported to be efficacious in patients with constipation-predominant irritable bowel syndrome by relieving gastrointestinal symptoms (Evans et al., 2004; Lesbros-Pantoflickova et al., 2004). Thus, several 5-HT_4 receptor agonists have proven to be clinically effective in the patients with gastrointestinal disorders.

Notably, further characterization of these agents has revealed that 5-HT_4 receptor agonists also have biologically relevant binding affinities for other 5-HT receptors as well as the dopamine (D_2) receptor, indicating that these agents are not highly selective 5-HT_4 receptor agonists. To this end, cisapride exhibits weak 5-HT_3 receptor antagonist properties as well as substantial affinity for the 5-HT_2 and D_2 receptors (Briejer et al., 1995; Karasawa et al., 1990). Mosapride likewise exhibits binding affinities for the 5-HT_{2B} and D_2 receptors which are similar to that for 5-HT_4 receptors (Mikami et al., 2008a). Tegaserod has been shown to be a potent 5-HT_{2B} receptor antagonist (Beattie et al., 2004). Moreover, it is noteworthy that, among these 5-

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HT_4 receptor agonists, cisapride and tegaserod were withdrawn from the market. Cisapride was withdrawn due to adverse cardiovascular effects associated with QT prolongation[1] because it is a potent blocker of the hERG (human ether-a-go-go-related gene) channel (Mohamand et al., 1997). Tegaserod has also been withdrawn from the market in response to a safety analysis indicating that patients treated with this drug had a higher chance of cardiovascular events than did those who were treated with placebo (Thompson, 2008).[2] Therefore, to date, no prokinetic agent is currently available to meet the unmet medical need resulting from the removal of these two drugs.

Recently, we have developed PF-01354082 a highly selective 5-HT_4 receptor partial agonist, PF-01354082, with a novel chemical structure, 4-{[4-({[(3-Isopropyl-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)carbonyl]amino}methyl)piperidin-1-yl]methyl}tetrahydro-2*H*-pyran-4-carboxylic acid (Kojima et al., 2008). This compound possesses benzimidazolone carboxylic acid structure that is different from those of current 5-HT_4 receptor agonists, cisapride and mosapride (benzamide), prucalopride (benzofran), and tegaserod (indole) (De Maeyer et al., 2008; Langlois and Fischmeister, 2003). In the present study, we report the pharmacological profile of PF-01354082 demonstrating that this novel compound is a highly selective partial agonist of the 5-HT_4 receptor having no effect on I_{Kr} current amplitude at a concentration 150,000-fold higher than the Ki for the human 5-HT_{4d} receptor. Moreover, in an in vivo study in fasted dogs, oral dosing of PF-01354082 was found to produce a long-lasting gastroprokinetic effect. These results indicate that PF-01354082 may be an effective clinical agent for the treatment of gastro-esophageal reflux disease and functional dyspepsia.

2. Materials and methods

2.1. Animals

Male CD IGS rats were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). Male beagle dogs were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies.

2.2. Receptor binding assays

Receptor binding assays for the human 5-HT receptors 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{3A}, 5-HT_{4d}, 5-HT_7, the dog 5-HT_{4h} receptor and the human dopamine D_{2long} receptor were performed using membrane preparations from HEK293 or CHO cells expressing either human or dog receptor. All cell membranes except for CHO cells expressing human 5-HT_{2A} receptors (Euroscreen, Brussels, Belgium) and 5-HT_7 receptors (PerkinElmer, Waltham, MA) were prepared in house. The cells expressing each receptor were homogenized in buffer on ice and then the homogenates were centrifuged (40,000–48,000 $\times g$). Homogenate buffers used in the study were as follows: 50 mM Tris–HCl buffer (pH 7.7) supplemented with a protease inhibitor (Sigma-Aldrich, St. Louis, MO) for human 5-HT_{1A}; 50 mM Tris–HCl buffer (pH 7.4), 2 mM $MgCl_2$ and a protease inhibitor cocktail (Roche Applied Bioscience, Indianapolis, IN) for 5-HT_{1B} and 5-HT_{1D}; 50 mM Tris–HCl buffer (pH 7.5) supplemented with the protease inhibitor for 5-HT_{3A}; 50 mM Tris–HCl buffer (pH 7.4) supplemented with the protease inhibitor for 5-HT_{4d}; 20 mM HEPES buffer (pH 7.4), 120 mM NaCl, 1 mM EDTA, and 1 mM EGTA for D_{2long}; 50 mM Tris–HCl buffer (pH 7.4), 10 mM $MgCl_2$ and the protease inhibitor for dog 5-HT_{4h}. The remaining pellets were suspended in the buffers as described below: 50 mM Tris–HCl buffer (pH 7.7), 4 mM $CaCl_2$, and 10 μM pargyline for 5-HT_{1A}; 50 mM Tris–HCl buffer (pH 7.4), 4 mM $CaCl_2$ for 5-HT_{1B} and 5-HT_{1D}; 50 mM Tris–HCl buffer (pH 7.5) for 5-HT_{3A}; 50 mM Tris–HCl buffer (pH 7.4) and 10 mM $MgCl_2$ for 5-HT_{4d}; 20 mM HEPES buffer (pH 7.4), 120 mM NaCl, 1 mM EDTA, and 1 mM EGTA for D_{2long}; 50 mM Tris–HCl buffer (pH 7.4) and 10 mM $MgCl_2$ for dog 5-HT_{4h}. Protein concentration was determined using a BCA protein assay kit (PIERCE, Rockford, IL). The following assay buffers were used for each receptor binding assay; 50 mM Tris–HCl (pH 7.7), 4 mM $CaCl_2$ and 10 μM pargyline for 5-HT_{1A}, 50 mM Tris–HCl (pH 7.4), 4 mM $CaCl_2$, 0.1% L-ascorbic acid and 10 μM pargyline for 5-HT_{1B} and 5-HT_{1D}, 50 mM Tris–HCl (pH 7.5) for 5-HT_{2A} and 5-HT_{3A}, 50 mM Tris–HCl buffer (pH 7.4) 10 mM $MgCl_2$ and 10 μM pargyline for 5-HT_{4d}, 50 mM Tris–HCl (pH 7.4), 10 mM $MgSO_4$, and 0.5 mM EDTA for 5-HT_7, 20 mM HEPES buffer (pH 7.4), 120 mM NaCl, 1 mM EDTA and 1 mM EGTA for D_{2long}; 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, and 20 μM pargyline for dog 5-HT_{4h}. The following radioligands were used for each receptor binding assay; [^{3}H]8-hydroxy-2-(di-*n*-propylamino) tetralin for 5-HT_{1A}, [^{3}H]5-HT for 5-HT_{1B}, 5-HT_{1D}, 5-HT_{4d} and dog 5-HT_{4h}, [^{3}H]BRL-43694 (granisetron) for human 5-HT_{3A}, [^{3}H]GR113808 for dog 5-HT_{4h}, [^{3}H]5-carboxamidotryptamine for 5-HT_7, and [^{3}H] spiperone for D_{2long}. All binding assays except those for 5-HT_{1A}, 5-HT_{3A}, and 5-HT_7 were performed by a filtration method using Whatman GF/B filtermat (PerkinElmer). 5-HT_{1A}, 5-HT_{3A}, and 5-HT_7 binding assays were carried out using WGA-SPA beads (Amersham plc, Buckinghamshire, UK).

2.3. 5-HT_4 agonistic activity on tunica muscularis mucosae (TMM) preparations from the rat isolated esophagus

Male CD IGS rats were euthanized by isoflurane inhalation and a 2 cm segment of intrathoracic esophagus was excised and placed in Krebs' solution (119 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.1 mM Glucose and 1.3 mM $CaCl_2$, pH 7.4). The external muscularis propria containing the outer longitudinal and circular muscle layers of the esophagus was carefully removed to isolate the TMM as described (Baxter et al., 1991). The strips were suspended in a 10 ml organ bath containing Krebs' solution at 37 °C aerated with 95% O_2 and 5% CO_2, under 0.5 g tension and equilibrated for 30 min. During the equilibration, indomethacin (3 μM), ketanserin (1 μM), and ascorbic acid (120 μM) were added to the Krebs' solution. Concentration–response curves were performed after contracting the rat TMM with carbachol (10 μM). Responses were measured isometrically using a TB-612T transducer (Nihon Kohden, Tokyo, Japan) coupled to a PowerLab data acquisition system (ADInstruments Inc., Colorado Springs, CO). Two concentration–response curves were performed per tissue: the first being a response to 5-HT and the second to the novel compound, PF-01354082. Agonist activity was confirmed by pre-incubating tissues with 1 μM SB203186, a 5-HT_4 receptor antagonist, which was added to the bath 5 min before the addition of carbachol.

2.4. 5-HT_4 agonistic activity on cAMP assay using cells expressing human 5-HT_{4d} receptors

The method utilized for the cAMP assay to determine 5-HT_4 agonistic activity in cells expressing human 5-HT_{4d} receptors was described elsewhere (Mikami et al., 2008b). Briefly, cells expressing the human 5-HT_{4d} receptor were used for the functional assay. Intracellular cAMP production was measured by a cAMP cell-based assay kit using HTRF® technology (Cisbio, Bagnols/Cèze Cedex, France). At the beginning of the assay, cell pellet was resuspended in DMEM supplemented with 20 mM HEPES, 10 μM pargyline, and 1 mM 3-isobutyl-1-methylxanthine and incubated for 15 min. The reaction was initiated by addition of the cells into plates containing

[1] FDA Talk Paper. Food and Drug Administration. U.S. Department of Health and Human Services. Janssen Pharmaceuticals stops marketing cisapride in the US. T00-14, March 23, 2000. http://www.fda.gov/bbs/topics/ANSWERS/ANS01007.html.

[2] Novartis Pharmaceutical Corporation, Media release. http://www.zelnorm.com/Zelnrom_PR_US_330_Final_12_1007.pdf.

serially diluted test compounds. After incubation for 15 min, 1% Triton X-100 was added to stop the reaction. A cAMP-XL665 conjugate was added to the lysate and then the anti-cAMP-cryptate conjugate was added. After further incubation for 60 min, measurements were made on a Wallac 1420 ARVOsx multilabel counter (excitation = 320 nm, emission = 665 nm/620 nm, delay time = 50 μs, window time = 400 μs). All experimental steps were done at room temperature.

2.5. hERG channel blocking assay

An experimental method for hERG (human either-a-go-go-related gene) was described elsewhere (Toga et al., 2007). HEK293 cells stably expressing hERG potassium channels licensed from Wisconsin Alumni Research foundation (Zhou et al., 1998.) were used in the experiment. The cells were superfused with a standard external solution (130 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 5 mM HEPES, adjusted pH 7.4 with NaOH). Whole-cell recordings were made using an EPC-9 patch-clamp amplifier controlled by the Pulse/PulseFit software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and patch pipettes which had a resistance of 1–3 MΩ when filled with an internal solution (130 mM KCl, 5 mM MgATP, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA, adjusted pH 7.2 with KOH). Whole-cell potassium currents through hERG channels were elicited by 1 s duration stepped depolarization pulse from a holding potential of −80 mV to +40 mV, followed by a descending repolarization ramp (0.5 mV ms^{-1}) back to the holding potential. The voltage pulse was applied to a cell continuously throughout the experiment every 4 s (0.25 Hz) to eliciting a large outward current. All experiments were carried out at 23 ± 1 °C. Test compounds were dissolved in DMSO and diluted with external solution [final concentration of DMSO was 0.5% (v/v)] and then applied to the cells. Percent decrease of I_N in each separate experiment was obtained by the normalized current value using following formula: $I_N = (1 - I_D/I_C) \times 100$, where I_D is the calculated mean value of drug responses. I_C is the mean value of the control currents. Data were expressed as mean ± S.E.M. from the independent I_N.

2.6. Measurement of the gastric motility in conscious dogs

Dogs were anesthetized with isoflurane and the abdominal cavity was opened under aseptic conditions. Extraluminal force transducers (F-12IS, Star Medical, Tokyo, Japan) were sutured onto the seromuscular layer of the gastric antrum, [3 cm proximal to the pyloric ring (modified from the method of Itoh et al., 1977)], the gastric body (10–15 cm proximal to the pyloric ring), the duodenum (10 cm distal to the pyloric ring), and the proximal colon (5 cm distal to the ileocecum). The lead wires of these transducers were taken out of the abdominal cavity and then brought out through a skin incision made between the scapulae. After surgery, protective jackets were placed on the dogs, and they were housed in individual cages. Recording of gastric motility was started at least 2 weeks after surgery. To start the experiment, the dogs were fasted overnight and placed in a shielded room, and then recording of gut motility in the fasted state was initiated. The motility was measured with a telemetry system (GTS-800, Star Medical, Tokyo, Japan) and data acquired into a personal computer with the acquisition software (Eight Star, Star Medical). After confirmation of the incidence of interdigestive migrating complex (IMC) at regular intervals, 0.5% methylcellulose (vehicle for PF-01354082) or PF-01354082 (0.0003, 0.001, 0.003, 0.01 or 0.03 mg/kg) was administered orally. Gut motility was then recorded for 6 h. To quantify gastric motility, the areas of the contractions of the gastric antrum were determined by the processing software (Analyze II, Star Medical, Tokyo). The area surrounded by the contraction curve and the baseline for every 2 h period after administration was calculated. For standardization, the calculated areas were divided by the peak height of the last IMC before administration, and used as the motor index (modified from the method of Sato et al., 2000). All results were presented as the mean ± S.E.M. Statistical analysis was performed with Dunnett's test using JMP software (version: 5.0.1 J, SAS Institute Inc., Cary, NC). Probability values of <0.05 were considered significant.

Table 1
Binding affinities of PF-01354082 and cisapride for human 5-HT_{4d} and dog 5-HT_{4h} receptors.

Receptors	Species	Ki (nM)	
		PF-01354082	Cisapride
5-HT_{4d}	Human	2.0 (1.9, 2.0)	79 (60, 100)
5-HT_{4h}	Dog	4.2 (3.2, 5.5)	16 (12, 23)

Values are geometric mean ($n = 3$) and values in parentheses are ±95% Confidence Interval. Ki values were calculated from the Cheng Prusoff equation.

Table 2
Agonistic activities of PF-01354082 and cisapride in functional assays using HEK 293 cells expressing the human 5-HT_{4d} receptor and rat esophageal TMM tissue.

Compound	cAMP elevation in HEK 293			
	Expressing human 5-HT_{4d} receptors		Relaxation of rat TMM	
	EC_{50}: nM	Emax: %	EC_{50}: nM	Emax: %
PF-01354082	3.4 (3.0, 3.8)	66 ± 6.4	7.2 (5.3, 9.8)	74 ± 5.9
Cisapride	140 (58, 330)	98 ± 7.2	49 (45, 53)	82 ± 1.3

Values are geometric mean ($n = 3$ or 4) and values in parentheses are ±95% Confidence Interval. For Emax, values are the mean ± S.E.M.

2.7. Chemicals

PF-01354082,
4-{[4-({[(3-Isopropyl-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl) carbonyl]amino}methyl)piperidin-1-yl]methyl}tetrahydro-2*H*-pyran-4-carboxylic acid, was synthesized by Global Research & Development, Nagoya Laboratories, Pfizer Japan Inc. Other chemicals used in this study were purchased commercially.

3. Results

3.1. Binding affinity and functional activity of PF-01354082 for 5-HT_4 receptors

To determine the affinity of PF-01354082 for human 5-HT_{4d} and dog 5-HT_{4h} receptors, PF-01354082 was evaluated in the receptor binding assay. Table 1 summarizes the binding affinities of PF-01354082 and cisapride for human 5-HT_{4d} and dog 5-HT_{4h} receptors. PF-01354082 displayed high affinity for both human 5-HT_{4d} and dog 5-HT_{4h} receptors illustrated by the Ki values of 2.0 nM [95%

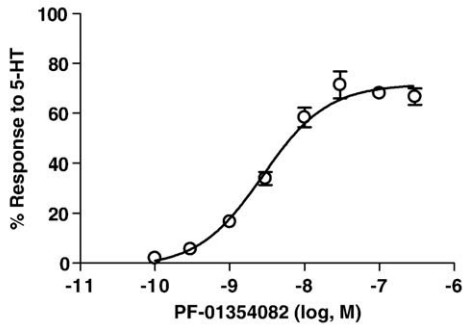

Fig. 1. Effect of PF-01354082 on accumulation of intracellular cAMP in HEK293 cells expressing human 5-HT_{4d} receptors. The *Y* axis is expressed as the percentage of 5-HT maximal response at 1000 nM. Data for % response to 5-HT were indicated by the mean ± S.E.M. ($n = 3$).

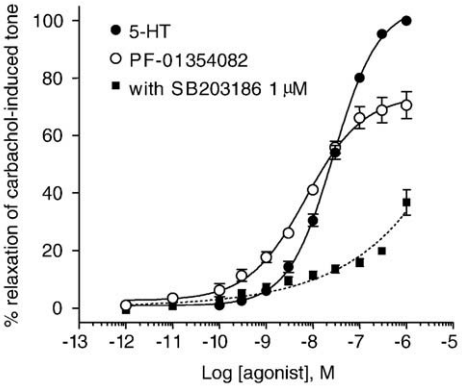

Fig. 2. Effect of PF-01354082 on relaxation of the rat esophageal TMM. PF-01354082 was subjected the assay in the presence (closed square) or in the absence (open square) of 5-HT_4 receptor antagonist SB203186 (1 μM). 5-HT (open circle) was also subjected the assay. Data were indicated by mean ± S.E.M. ($n = 3$). Concentration–effect curves were obtained after contracting the rat TMM with 10 μM of carbachol.

Confidence Interval (CI): 1.9–2.0 nM] and 4.2 nM (95% CI: 3.2–5.5 nM), respectively. Notably, the Ki values of PF-01354082 were markedly lower for both receptors than those of cisapride indicating a greater affinity for the novel compound. Next, the functional activities of PF-01354082 and cisapride were evaluated using cells expressing human 5-HT_{4d} receptors (Table 2 and Fig. 1). PF-01354082 stimulated cAMP production in the cells with an EC_{50} of 3.4 nM (95% CI: 3.0–3.8 nM) and an Emax of 66% ± 6.4 (mean ± S.E.M.) relative to 5-HT. Functional activity was also assessed in a native receptor from the rat TMM. PF-01354082 similarly exhibited potent efficacy for muscle relaxation in this preparation with an EC_{50} of 7.2 nM (95% CI: 5.3–9.8 nM) and an Emax of 74% ± 5.9 (mean ± S.E.M.) relative to 5-HT (Fig. 2). Furthermore, we demonstrated that the relaxation caused by PF-01354082 could be inhibited in the presence of SB-203186, a selective 5-HT_4 receptor antagonist, confirming that the relaxation by in response to PF-01354082 was mediated by via activation of 5-HT_4 receptors. These results indicate that PF-01354082 is a potent 5-HT_4 receptor partial agonist. By contrast, the potency of cisapride for both the human and rat 5-HT_{4d} receptors as measured in these same assays was markedly lower than those of PF-01354082 (Tables 1 and 2).

3.2. Selectivity of PF-01354082 for various receptor and transporter binding in vitro

Next, we investigated the selectivity of PF-01354082 for other human 5-HT and D_{2long} receptors in vitro. A shown in Table 3, PF-01354082 did not exhibit significant affinity for any of the other receptors tested (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{3A}, 5-HT_7, and D_{2long} receptors), showing >1800-fold selectivity for human 5-HT_{4d} receptor over these receptors. In contrast, cisapride displayed higher affinity for both the human 5-HT_{2A} and the D_{2long} receptors as indicated by the fact that the Ki values of cisapride for were lower for these receptors (3 and 25 nM, respectively) than that determined for the 5-HT_{4d} receptor (79 nM, Table 2). Furthermore, cisapride showed moderate binding affinities for human 5-HT_{1A}, 5-HT_{1D}, and 5-HT_{3A} receptors (Table 3).

We also investigated the effects of PF-01354082 on the in vitro activity of various other receptors, ion channels, and uptake sites (Table 4). When tested at a final concentration of 10 μM, PF-01354082 did not show relevant biological activity except for the 5-HT_{4e} receptor which resulted in 100% inhibition. Further examination revealed that PF-01354082 had a potent binding affinity for the 5-HT_{4e} receptor yielding a Ki value of 10 nM (data not shown). However, 10 μM PF-01354082 did not inhibit biological activity at other 5-HT receptors such as the 5-HT_{2B} and 5-HT_{2C} receptors, confirming that PF-01354082 is highly selective for 5-HT_4 receptors.

3.3. PF-01354082 stimulated gastric motility in conscious dogs

PF-01354082 was assessed for its ability to increase gut motility in the fasted state using conscious dogs instrumented with strain gauge force transducers in the gastric antrum. As shown in Fig. 3, oral administration of PF-01354082 at doses of 0.0003–0.03 mg/kg resulted in marked and long-lasting stimulation of gastric motility in a dose-dependent manner. During the 0 to 2 h period after oral administration of PF-01354082, the measured change in the motor index achieved statistical significance at a dose of 0.001 mg/kg, which was determined to be the minimum effective dose in this model.

3.4. Effect of PF-01354082 on hERG channels

Finally, the effect of PF-01354082 on cellular potassium current was investigated using HEK293 cells transfected to express hERG channels. PF-01354082 decreased I_{HERG} current by 11 ± 1.1% (mean ± S.E.M., $n = 4$) at a concentration of 300 μM. This indicates that PF-01354082 showed greater than 150,000-fold selectivity for the human 5-HT_{4d} receptor over the hERG channel.

4. Discussion

In this study, we investigated the in vitro pharmacological profile of PF-01354082 and its effect on gastric motility in conscious dogs. Binding affinity and functional activity of PF-01354082 for 5-HT_4 receptors and other receptors revealed that PF-01354082 is a potent partial agonist at the 5-HT_4 receptor exhibiting a high degree of selectivity against other 5-HT receptors, the D_2 receptor, and various other receptors we evaluated. Based on a conscious dog in vivo study, oral administration of PF-01354082 is capable of producing a sustained gastroprokinetic effect in the fasted state. Recently, we developed another novel 5-HT_4 partial agonist, CJ-033,466 (Mikami et al., 2008a). This compound was a potent agonist for the 5-HT_4 receptor, but also exhibited weak affinities for human 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, and human D_{2long} receptors. By contrast, PF-01354082 displays more than 3000-fold selectivity for human 5-HT_{4d} receptor over these other 5-HT receptors and more than 1800-fold selectivity over the D_{2long} receptor, indicating that PF-01354082 has better selectivity for human 5-HT_4 receptors compared to that of CJ-033,466. Furthermore, CJ-033,466 was found to have potential phototoxicity (Onoue et al., 2008a,b). CJ-033,466 has imidazopyridine moiety in its core chemical structure and the presence of imidazopyridine moiety as part of a drug molecule was considered a structural alert for drug-induced phototoxicity. In contrast, a very low light absorption in the region of 290–700 nm was observed in a compound having benzimidazolone moiety as a core chemical structure that is the same as PF-01354082, and furthermore, this compound was

Table 3
Binding affinities of PF-01354082 and cisapride for human 5-HT and dopamine receptors.

Receptors	Ki (nM)	
	PF-01354082	Cisapride
5-HT_{1A}	>5000	700 (500, 970)
5-HT_{1B}	>5500	>5,100
5-HT_{1D}	>6200	680 (520, 870)
5-HT_{2A}	>6100	3.1 (2.7, 3.5)
5-HT_{3A}	>4900	800 (630, 1,000)
5-HT_7	>4000	>4100
D_{2long}	>3700	25 (18, 34)

Values are geometric mean ($n = 3$ or 4) and values in parentheses are ±95% Confidence Interval. Ki values were calculated from the Cheng Prusoff equation.

Table 4
The effects of 10 μM PF-01354082 on in vitro binding to selected receptors, ion channels, and transporters.

Receptor/Transporter/Ion channels		% Inhibition	Reference compound	
	Subtype	PF-01354082	Compound name	IC_{50} (nM)
Adenosine	A_1 (human)	–*	1,3-Dipropy-8-cyclopentylxanthine	27
	A_{2A} (human)	–	5'-*N*-ethylcarboxamidoadenosine	43
	A_3 (human)	–	IB-MECA	3.1
Adrenergic	α_1 (non-selective)	–	Prazosin	0.52
	α_{2A} (human)	–	Yohimbine	3.6
	α_{2B}	–	Yohimbine	8.9
	β_1 (human)	–	Atenolol	370
	β_2 (human)	31	ICI 118551	13
	β_3 (human)	–	Cyanopindolol	41
Angiotensin II	AT_1 (human)	–	Saralasin	0.69
Benzodiazepine	(central)	13	Diazepam	23
Bradykinin	B_2 (human)	–	NPC 567	5.6
Cannabinoid	CB_1 (human)	13	WIN 55212-2	11
	CB_2 (human)	–	WIN 55212-2	3.9
Cholecystokinin	CCK_A (human)	–	CCK-8	1.5
	CCK_B (human)	–	CCK-8	2.3
Component 5a	(human)	–	hC5a	0.13
Dopamine	D1 (human)	–	SCH 23390	0.57
	D2S (human)	–	(+) Butaclamol	17
	D3 (human)	16	(+) Butaclamol	10
Endothelin	ET_A (human)	–	Endothelin-1	0.099
	ET_B (human)	–	Endothelin-3	0.11
GABA	$GABA_A$	10	Muscimol	28
	$GABA_B$	–	Baclofen	52
Glutamate	α-Amino-3-hydroxy-5-methyl-4-isoxazelepropionic acid			
	Kainate	–	Kainic acid	38
	N-Methyl-d-aspartate	–	CGS 19755	1000
Glycine	Glycine (strychnine-sensitive)	–	Strychnine	7.0
	Glycine (strychnine-insensitive)	–	Glycine	250
Histamine	H_1 (central)	24	Pyrilamine	4.5
	H_2	–	Cimetidine	710
	H_3	–	(R)-α-Me-histamine	2.4
Monoamine and neurotransmitter synthesis and metabolism				
	MAO-A	–	Clorgyline	2.2
	MAO-B	–	(R)-Deprenyl	10
Muscarinic	M_1 (human)	–	Pirenzepine	32
	M_2 (human)	–	Methoctramine	31
	M_3 (human)	–	4-DAMP	0.59
Nicotinic	Neuroral (α-bungarotoxicin-insensitive)			
		–	Nicotine	11
	Muscle-type (human)	–	α-Bungarotoxin	9.5
Opioid and opioid-like				
	δ_2 (human) (DOP)	–	DPDPE	4.2
	κ (KOP)	–	U 50488	2.1
	μ (human) (MOP)	10	DAMGO	11
	ORL1 (human) (NOP)	–	Nociceptin	5.4
Serotonin	$5\text{-}HT_{1A}$ (human)	–	8-OH-DPAT	2.2
	$5\text{-}HT_{1B}$	19	Serotonin	14
	$5\text{-}HT_{2A}$ (human)	–	Ketanserin	4.3
	$5\text{-}HT_{2B}$ (human)	–	Serotonin	110
	$5\text{-}HT_{2C}$ (human)	–	SB 242084	1.8
	$5\text{-}HT_{4e}$ (human)	100	Serotonin	300
	$5\text{-}HT_7$ (human)	–	Serotonin	0.5
Somatostatin	sst_5 (human)	–	Somatostatin	0.52
Steroid nuclear receptor				
	Glucocorticoid (human)	–	Dexamethasone	1.6
Non-steroid nuclear receptor				
	TR (TH)	–	T3	0.44
Urotensin-II	UT	–	Urotensin-II	2.6
Vasopressin	$V_1\alpha$ (human)	10		$[d(CH2)^1_5$, Tyr $(Me)^2$]-AVP 2.1
Ca^{2+} channel	L-type, Dihydropyridine site	–	Nitrendipine	2.0
	L-type, diltiazem site	10	Diltiazem	28
	L-type, verapamil site	–	D 600	25
	N-type	–	ω-Conotoxin GVIA	0.0095
K^+ channel	K^+_{ATP} channel	–	Glibenclamide	3.5
Na^+ channel	Site 2	13	Veratridine	4400
Transporter	Norepinephrine transporter (human)	–	Protriptyline	11
	Dopamine transporter (human)	–	BTCP	18
	GABA transporter	–	Nipecotic acid	3400
	Choline transporter	16	Hemicholinium-3	6.7
	5-HT transporter (human)	–	Imipramine	3.3

*: –, inhibition of less than 10%.
The results are expressed as a percentage of inhibition of control specific binding (mean values, $n = 2$).
The IC_{50} values were determined by non-linear regression analysis of the competition curves using Hill equation curve fitting.

photochemically stable (data not shown). These data suggest that there is only a low risk of PF-01354082 having phototoxic potential because PF-01354082 is a different chemotype to CJ-033,466.

Several $5\text{-}HT_4$ receptor agonists including cisapride, mosapride, and tegaserod have been developed for gastrointestinal motor disorders and these agents have been shown to be efficacious for patients with gastrointestinal motor disorders, such as gastro-esophageal reflux disease and irritable bowel syndrome (Evans et al., 2004; Kahrilas et al., 2000; Lesbros-Pantoflickova et al., 2004; Maddern et al., 1991; Pehlivanov et al., 2002; Rodoriguez-Stanley et al., 2006; Ruth et al., 1998, 2003; Wienbeck and Li, 1989). However, these agents are not highly selective and exhibit some affinity for not only other 5-HT receptors (in addition to $5\text{-}HT_4$ subtype) but also for other neurotransmitter receptors including dopamine D_2 receptors. Cisapride has a weak $5\text{-}HT_3$ receptor antagonist property as well as reasonable affinity for $5\text{-}HT_2$, D_2, α_1 adrenoreceptor and muscarinic receptors (Briejer et al., 1995; Holtmann and Talley, 1993; Karasawa et al., 1990). Mosapride exhibits binding affinities for $5\text{-}HT_{2B}$ and D_2 receptors which are similar to that of the $5\text{-}HT_4$ receptors and shows a weak binding affinity for $5\text{-}HT_{2A}$ receptors (Mikami et al., 2008a). Likewise tegaserod is a potent $5\text{-}HT_{2B}$ receptor antagonist (Beattie et al., 2004) with additional potent binding affinities for $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, $5\text{-}HT_{1D}$, and $5\text{-}HT_{2A}$ receptors comparable to that for $5\text{-}HT_{4d}$ receptors as well as weak binding affinities for $5\text{-}HT_7$ and D_2 receptors (Mikami et al., 2008a). Together these data indicate that tegaserod is a non-selective $5\text{-}HT_4$ receptor agonist. Among 5-HT receptors, $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$, $5\text{-}HT_4$, and $5\text{-}HT_7$ receptors and D_2 receptors have been shown to have a possible role in the function of the gastrointestinal tract (Spiller, 2001; Willems et al., 1985). For instance, $5\text{-}HT_4$ and $5\text{-}HT_7$ receptors mediate relaxation in isolated human colonic circular muscle (Prins et al., 1999; Tam et al., 1995) and $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptors modulate gastric emptying in a cooperative way (Borman et al., 2002; Komada and Yano, 2007; McCullough et al., 2006). $5\text{-}HT_3$ receptors are reported to play a role in mediating the activation of mucosal terminals of myenteric intrinsic primary afferent neurons that initiate the peristaltic reflex, indicating that $5\text{-}HT_3$ antagonists slow intestinal motility (Bertrand et al., 2000). Furthermore, $5\text{-}HT_1$ receptor agonist causes a relaxation of the gastric fundus and inhibition of antral contractile activity (Coulie et al., 1997). Given

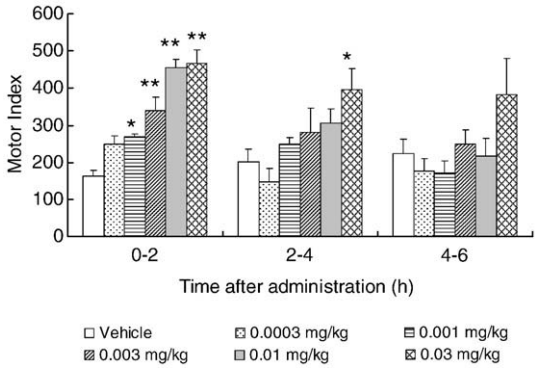

Fig. 3. The effect of orally administered PF-01354082 on the gastric motility in the fasted state in conscious dogs. Data were indicated by mean ± S.E.M. ($n = 4$). *: $p<0.05$, **: $p<0.01$, versus vehicle treatment group (Dunnett's test).

that the 5-HT_4 receptor agonists, cisapride, mosapride, and tegaserod also exhibit affinities for other 5-HT receptors and D_2 receptors, the demonstrated efficacy of these agents on gastrointestinal motor disorders may be mediated by a combination of effects through multiple receptors. Alternatively, their prokinetic effects via 5-HT_4 receptor activation are possibly reduced by additional agonistic activities for 5-HT_1 receptors or antagonizing activities for 5-HT_2 and 5-HT_3 receptors that play roles in reducing gastrointestinal motility. Furthermore, modulation of other 5-HT receptors and D_2 receptors may give rise to the risk of adverse effects. The antagonism of central D_2 receptors induces adverse effects including hyperprolactinaemia and extrapyramidal dystonic reactions (Tonini et al., 2004). Additionally, a selective 5-HT_3 receptor antagonist, alosetron, has been reported to be associated with ischemic colitis and serious complications of constipation (Mayer and Bradesi, 2003). This relationship has been further confirmed with additional data of more recent clinical trials (Chang et al., 2006) although the mechanism of 5-HT_3 receptor antagonist induced-ischemic colitis or serious complications of constipation have not fully been elucidated (Camilleri, 2007; Gallo-Torres et al., 2006). Similarly, the mechanism of action of current 5-HT_4 receptor agonists on gastrointestinal motility and also their potential risk for adverse effects by modulating other 5-HT and neurotransmitter receptors also still remains to be elucidated. Taken together, 5-HT_4 receptor agonists (cisapride, mosapride, and tegaserod) are not highly selective for 5-HT_4 receptors and exhibit some affinity for not only other 5-HT receptors but also for other neurotransmitter receptors. This non-selectivity may influence the agent's safety, therapeutic potential for the treatment of GI motility disorders, and overall benefit/risk profile. The clinical development of these non-selective 5-HT_4 receptor agonists has been complicated by the occurrence of adverse effects and these effects have influenced the perception on the use of 5-HT_4 receptor agonists in GI motility disorders (De Maeyer et al., 2008). In contrast to the current 5-HT_4 receptor agonists, PF-01354082 is highly potent and selective for the 5-HT_4 receptor and shows no affinity for other 5-HT receptors (5-HT_1, 5-HT_2 and 5-HT_3) or D_2 receptors. Furthermore, no metabolism of PF-01354082 was observed in human microsomes (data not shown), indicating that PF-01354082 is metabolically stable compound. Several benzimidazolone derivatives possess 5-HT_3 receptor antagonist or 5-HT_4 receptor antagonist properties (Langlois and Fischmeister, 2003). Metabolic stability of PF-01354082 may eliminate the possibility of active metabolites generated from PF-01354082 possessing 5-HT_3 receptor antagonist or 5-HT_4 receptor antagonist properties. Given that 5-HT_4 receptor agonists have clear-cut prokinetic effects in the GI tract (Gershon and Tack, 2007; Talley, 2002; Tonini and Pace, 2006), this improved binding profile provides substantial evidence that PF-01354082 may provide better clinical efficacy with reduced adverse effects mediated by off target interactions with other receptors as compared to the current non-selective 5-HT_4 receptor agonists. In fact, prucalopride, a highly selective 5-HT_4 receptor agonist, significantly and consistently improved bowel function, associated symptoms and satisfaction in the patients with severe chronic constipation (Camilleri et al., 2008; Quigley et al., 2009; Tack et al., 2009). Larger and long-term clinical trials are required to fully access the risks and benefits of the use of a selective 5-HT_4 receptor agonist to patients with GI motility disorders.

Cisapride, a 5-HT_4 receptor agonist, had been marketed for gastrointestinal prokinetics, however, it was withdrawn due to potent hERG activity and QT prolongation. (Mohamand et al., 1997; Rampe et al., 1997; Toga et al., 2007; Walker et al., 1999). Evaluation of PF-01354082 in HEK293 cells expressing hERG channels revealed that this novel compound showed only a small effect of 11% of inhibition at 300 μM. The hERG channel is a primary potassium channel responsible for the repolarization phase of the cardiac action potential (Curran et al., 1995; Sanguinetti et al., 1995) and is routinely used a tool for the identification of potential high risk compounds causing a delay in ventricular repolarization and prolongation of the QT interval by ICH S7B guideline[3]. Our result showing low inhibitory activity of PF-01354082 for hERG channel supports that PF-01354082 possesses a safe profile for cardiac risk of QT prolongation compared to cisapride.

In this study, we also investigated the stimulatory effect of PF-01354082 in a gastric antral motility model using fasted dogs. Gastric motility in conscious dogs is divided into two patterns which are interdigestive state and digestive state. Gastric motility in the fasted state is characterized as the IMC that occurs at regular intervals in the gut. This pattern of gastric motility is similarly observed in human (Itoh and Sekiguchi, 1983). Involvement of 5-HT_4 receptors in contractile responses of gut in dogs has been reported. A selective 5-HT_4 receptor antagonist, SB204070, was previously shown to inhibit 5-HT-induced gastric constriction while another 5-HT_4 receptor agonist was observed to induce an atropine-sensitive gastric constriction in dogs (Bingham et al., 1995; Bermudez et al., 1990). It has also been shown that excitatory neuronal 5-HT_4 receptors are located in gastric antral muscles in dogs (Prins et al., 2001) further supporting that gastric antral motility is mediated by 5-HT_4 receptors in this species. In fact, we have previously reported that a highly selective 5-HT_4 receptor partial agonist stimulated gastric antral motility in fasted conscious dogs and also accelerated the gastric emptying rate in a dog model of gastroparesis (Mikami et al., 2008a). Given that 5-HT_4 receptors are located in the myenteric plexus and muscle layer of the antrum and the corpus of the human stomach (Sakurai-Yamashita et al., 1999), the gastric antral motility model in dogs is a good model to predict pharmacological efficacy of 5-HT_4 receptor agonists in humans. Indeed, oral administration of PF-01354082 to conscious dogs resulted in marked and long-lasting stimulation of gastric motility in a dose-dependent manner, clearly supporting that PF-01354082 should have clinical efficacy in the patients with gastric motility disorders.

In summary, we have developed PF-01354082 as an orally active, highly selective, partial 5-HT_4 receptor agonist that has minimal activity against other 5-HT and related receptors involved in gastrointestinal motility. Notably, PF-01354082 has a markedly improved safety margin for inhibitory activity against the hERG channel. In vivo studies in fasted conscious dogs showed that PF-01354082 stimulated gastric motility in a dose-dependent manner. Taken together, these promising results indicate that PF-01354082 should exert a favorable pharmacological effect on gastrointestinal motor disorders with reduced adverse effects mediated via other 5-HT and neurotransmitter receptors in the GI tract. And thus warrants

[3] ICH 7SB Guideline. The non-clinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. May 12, 2005. http://www.ich.org/LOB/media/MEDIA2192.pdf.

clinical evaluation of this compound in the patients with gastrointestinal motor disorders.

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