Full Paper

Evaluation of the Pharmacological Profile of Ramosetron, a Novel Therapeutic Agent for Irritable Bowel Syndrome

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Abstract. We examined the pharmacological profile of ramosetron, a 5-HT₃-receptor antagonist for irritable bowel syndrome with diarrhea, comparing it with those of other 5-HT₃-receptor antagonists, alosetron and cilansetron, and the anti-diarrheal agent loperamide. Ramosetron showed high affinity for cloned human and rat 5-HT₃ receptors, with K_i values of 0.091 ± 0.014 and 0.22 ± 0.051 nmol/L, respectively, while its affinities for other receptors, transporters, ion channels, and enzymes were negligible. Dissociation of ramosetron from the human 5-HT₃ receptor was extremely slow ($t_{1/2} = 560$ min), while alosetron ($t_{1/2} = 180$ min) and cilansetron ($t_{1/2} = 88$ min) dissociated relatively rapidly. Ramosetron competitively inhibited 5-HT-induced contraction of isolated guinea-pig colon, with pA_2 values of 8.6 (8.5–9.0). Ramosetron given orally also dose-dependently inhibited the von Bezold-Jarisch reflex in rats, with an ED_{50} value of 1.2 (0.93–1.6) $\mu\text{g/kg}$. In addition, oral ramosetron dose-dependently inhibited restraint stress-induced defecation in rats, with an ED_{50} value of 0.62 (0.17–1.2) $\mu\text{g/kg}$. In all of these experiments, the potencies of ramosetron were greater than those of alosetron, cilansetron, or loperamide. These results indicate that ramosetron is a highly potent and selective 5-HT₃-receptor antagonist, with beneficial effects against stress-induced abnormal defecation in rats.

Keywords: ramosetron, 5-HT₃ receptor, guinea-pig colon, von Bezold-Jarisch reflex, restraint stress

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an indoleamine that is widely distributed in nature. In humans, about 95% of the total 5-HT is found in the gut (1). It is well known that 5-HT plays important physiological roles in contraction/relaxation of smooth muscle, platelet aggregation, and neuronal transmission. Receptors mediating the actions of 5-HT are now classified into seven major groups, termed 5-HT₁ to 5-HT₇, which include a total of 14 subtypes (2). Among these receptors, the 5-HT₃ receptor possesses unique characteristics. This receptor is a ligand-gated cation channel that belongs to the nicotine/GABA-receptor superfamily, while all other 5-HT-receptor subtypes belong to the family of G-protein-coupled receptors (2).

It has been reported that 5-HT₃ receptors are widely distributed within the neurons of the GI tract as well as in the spinal cord and brain (2), and activation of 5-HT₃ receptors results in intestinal secretion and peristaltic activity (3, 4).

Irritable bowel syndrome (IBS) is a functional disease with persisting gastrointestinal symptoms, mainly abdominal pain/discomfort and defecation, not accompanied by an organic disease (5). The cause of IBS is unknown, but a number of factors such as altered gastrointestinal motility, increased sensitivity of the gut, psychosocial factors, and an imbalance in neurotransmitters are thought to play a role (6). In the last decade, the neurotransmitter 5-HT has received much attention as one of the pathogenic factors of IBS. Furthermore, 5-HT₃-receptor antagonists have been reported to inhibit abnormal defecation and to increase the perceptual threshold of the colon (7, 8), which suggested the involvement of 5-HT₃ receptors in the pathogenesis of IBS.

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Ramosetron, a potent 5-HT₃-receptor antagonist (9), has been launched in Japan as a medication for gastrointestinal symptoms caused by anti-tumor agents (10), and it is currently under development for use in patients suffering from IBS with diarrhea (IBS-D). Recently, several 5-HT₃-receptor antagonists such as alosetron (11) and cilansetron (12) have been developed for IBS-D. However, no study has been performed to directly compare the potencies of these drugs.

In the present study, therefore, we examined the affinity and the antagonistic activity of ramosetron for the 5-HT₃ receptor both *in vitro* and *in vivo*, in comparison with those of alosetron and cilansetron. In addition, we also examined the inhibitory effect of ramosetron on restraint stress-induced defecation in rats and compared it with those of alosetron, cilansetron, and loperamide.

Materials and Methods

Animals

Male Hartley guinea pigs weighing 650–840 g (Charles River Laboratories Japan, Inc., Kanagawa), male Wistar rats weighing 230–340 g (Japan SLC, Inc., Shizuoka and Clea Japan, Inc., Tokyo), and male Sprague-Dawley rats weighing 230–340 g (Clea Japan) were used. All animals were given food and water *ad libitum* and were housed in a temperature-controlled environment (22 ± 2°C) under a 12-h light/dark cycle. All animal experimental procedures were approved by the Animal Ethical Committee of Astellas Pharma, Inc. (Tokyo).

Drugs

In this study, ramosetron hydrochloride, alosetron hydrochloride, cilansetron hydrochloride (Astellas Pharma, Inc.); MDL72222, loperamide hydrochloride (Sigma-Aldrich Japan, Tokyo); and 5-HT creatinine sulfate (Wako Pure Chemical Industries Ltd., Osaka) were used. Ramosetron, alosetron, cilansetron, and MDL72222 were dissolved in distilled water and diluted with the vehicle in each experiment. Loperamide was suspended in and diluted with 0.5% (w/v) methyl cellulose (MC) solution. Compound 5-HT was dissolved and diluted with Krebs-bicarbonate solution or physiological saline. In this study, the dose levels of all test compounds except 5-HT were expressed as their hydrochloride form.

Affinities for the 5-HT₃ receptor

A membrane preparation of HEK293 cells expressing human recombinant 5-HT₃ receptors was purchased from Perkin Elmer (Waltham, MA, USA), and a mem-

brane preparation of COS-1 cells expressing rat cloned 5-HT₃ receptors was prepared using a modification of the method of Miyake et al. (13). Specifically, COS-1 cells were transfected with rat 5-HT₃ plasmid DNA using FuGENE (Roche Diagnostics, Indiana, USA) and were then incubated in Dulbecco's modified Eagles medium (Invitrogen, CA, USA) at 37°C under 5% (v/v) CO₂ for 3 days. Cells were removed and homogenized in 50 mmol/L Tris-HCl buffer and then centrifuged at 40,000 × *g*. The pellet was homogenized and recentrifuged, and the final pellet was suspended in 50 mmol/L Tris-HCl buffer for membrane preparation.

A receptor binding assay was performed according to the method of Akuzawa et al. (14). Aliquots (50 µL) of each test solution, or of 50 mmol/L Tris-HCl buffer, were added to 300 µL of 50 mmol/L Tris-HCl buffer and subsequently mixed with 50 µL of [³H]-GR65630 (specific activity: 2,245.9 GBq/mmol, Perkin Elmer) diluted to the required concentration. Aliquots (100 µL) of human or rat 5-HT₃-receptor membrane preparations at 2.8 or 100 µg protein/mL were added, and after thorough mixing of the samples (final assay volumes of 500 µL), reactions proceeded at room temperature for 1 h. Mixtures were then aspirated and filtered using a GF/B filter (Whatman, Kent, UK) and a cell harvester (MPR-24; Brandel, MA, USA). The filter was immediately washed three times with ice-cold 50 mmol/L Tris-HCl buffer. The filter was dried and cut out. Then, 5 mL of Aquasol-2 (Perkin Elmer) was added to the filter paper fragments, in a scintillation vial, and radioactivity levels measured using a liquid scintillation counter (TRI-CARB 2000CA, Perkin Elmer).

To test drug effects on receptor binding, ramosetron, alosetron, or cilansetron was added to the reaction mixtures described above to achieve final concentrations of 0.001–3, 0.03–100, and 0.03–100 nmol/L, respectively, at a common ratio of approximately 3 (three experiments, each in duplicate). Compound [³H]-GR65630 was added at final concentrations of 0.02–5 nmol/L in the saturation experiment (a single experiment, performed in duplicate) and at a final concentration of 0.5 nmol/L in experiments where test drugs were present. In place of the test compound, MDL72222 (final concentration: 10 µmol/L) was added to determine the non-specific binding of [³H]-GR65630. Such non-specific binding was deducted from the total binding in order to obtain a measure of specific binding.

Dissociation from the human 5-HT₃ receptor

Twenty-microliter aliquots of replacement substance solution or test compound solutions were added to tubes for calculation of non-specific binding or binding inhibition rates, respectively. After 60 µL of assay buffer and

20 μ L of receptor solution at 110 μ g protein/mL had been added to each tube, the mixtures were incubated at room temperature for 1 h. The mixtures were then centrifuged at $17,800 \times g$ for 5 min, and the supernatant was removed while the precipitate was retained. An 80- μ L aliquot of assay buffer was added to each tube to suspend the precipitate, and 100 μ L of 20 nmol/L [3 H]-GR65630 was added to each tube. After incubation at room temperature for 0–720 min, the mixtures were filtered through GF/C filters (Whatman) using a cell harvester, and the filter papers were rinsed three times with ice-cold 50 mmol/L Tris-HCl. A filter paper was placed in a vial, 5 mL of liquid scintillator (Atomlight, Perkin Elmer) was added, and radioactivity was counted using a liquid scintillation counter.

In this experiment, ramosetron, alosetron, and cilansetron were added to final concentrations of 1 nmol/L. In place of the test compound, MDL72222 (final concentration of 10 μ mol/L) was added to determine non-specific binding of [3 H]-GR65630. The assay was performed once in quadruplicate.

Affinity for other 5-HT-receptor subtypes

Other radioligand binding assays were performed as described below: [3 H]-8-hydroxy-2-(dipropylamino) tetralin (8-OH-DPAT) binding to human recombinant 5-HT_{1A} receptors, [125 I]-iodocyanopindolol binding to rat brain 5-HT_{1B} receptors, [3 H]-ketanserin binding to human recombinant 5-HT_{2A} receptors, [125 I]-lysergic acid diethylamide (LSD) binding to human recombinant 5-HT_{2B} receptors, [3 H]-mesulergine binding to human recombinant 5-HT_{2C} receptors, [3 H]-GR113808 binding to guinea pig 5-HT₄ receptors, [125 I]-LSD binding to human recombinant 5-HT_{5A} receptors, [125 I]-LSD binding to human recombinant 5-HT₆ receptors, and [125 I]-LSD binding to human recombinant 5-HT₇ receptors. The cell membrane preparation expressing 5-HT_{1B} or 5-HT₄ receptors were prepared from rat brain or guinea-pig striatum (obtained from Funakoshi Co., Ltd., Tokyo) by the methods previously reported (15, 16), whereas the other preparations were purchased from Perkin Elmer. In the first set of experiments, the inhibition rates of ramosetron, alosetron, and cilansetron, at final concentrations of 10 nmol/L, were evaluated (a single experiment, performed in duplicate). If an inhibition rate >50% was noted, replacement and saturation experiments were performed to assess the affinity of the test compound for the receptor (three experiments, performed in duplicate).

Affinities for other receptors, ion channels, transporters, and enzymes

The inhibition rate of ramosetron at a final concentra-

tion of 10 μ mol/L of 44 receptors, 5 ion channels, 3 transporters, and 3 enzymes was determined. The following receptors were examined: adenosine A₁ (rat)-, α_1 (rat)-, α_2 (rat)-, and β (rat)-adrenergic, angiotensin AT₁ (human) and AT₂ (human), bradykinin B₂ (human), cholecystokinin CCK-1 (human) and CCK-2 (human), corticotropin releasing factor CRF₁ (human), dopamine D₁ (rat) and D₂ short (human), estrogen (rat), endothelin ET_A (human) and ET_B (human), γ -aminobutyric acid GABA_A [the agonist site (rat) and the benzodiazepine site (rat) were examined], GABA_B (rat), glutamate [the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA, rat), kainite (rat), *N*-methyl-D-aspartic acid (NMDA) agonist (rat), and NMDA glycine receptor sites (rat) were examined], glycine (the strychnine-sensitive receptor, rat), histamine H₁ (central, guinea pig), H₂ (rat), and H₃ (rat), leukotriene B₄ (guinea pig) and D₄ (guinea pig), melatonin MT₁ (human), muscarinic non-selective (rat), M₁ (human), and M₂ (human), neurokinin NK₁ (human), NK₂ (human), and NK₃ (human), nicotinic Ni (neuronal, non-selective, rat), opiate non-selective (rat) and μ (human), oxytocin (rat), platelet activating factor PAF (rabbit), sigma (guinea pig), testosterone (human), vasopressin V₁ (rat), and vaso-active intestinal peptide VIP (human). The ion channels were the type L (dihydropyridine, rat) and the type N (rat) Ca²⁺ channel, the K⁺ channels K_{ATP} (rat) and SK_{Ca} (rat), and the Na⁺ channel (site 2, rat). The following transporters were examined: dopamine (human), norepinephrine (human), and 5-HT (human). The three enzymes acetylcholinesterase (electric eel), monoamine-oxidase-A (rat), and monoamineoxidase-B (rat) were also examined. The non-human receptors, ion channels, and enzymes were prepared from the animal tissues (Funakoshi Co., Ltd.) by the methods previously reported, and the human recombinant receptors and transporters were purchased from Perkin Elmer. Inhibition by ramosetron was evaluated using appropriate cell membrane preparations and enzymes and specific radiolabeled ligands (a single experiment, performed in duplicate). If the drug inhibition rate was >50%, the affinity of ramosetron for the receptor, ion channel, transporter, or enzyme was assessed (three experiments, performed in duplicate).

Inhibition of 5-HT-induced contraction of isolated guinea-pig distal colon

This experiment was performed according to the method of Miyata et al. (17). After guinea pigs were sacrificed by exsanguination, the distal colons were isolated. In an organ bath thermostatically controlled at 37°C, a distal colon specimen (approximately 2-cm-long) was vertically suspended in the direction of the

longitudinal muscle, under a loading tension of 1 g, in 10 mL of Krebs-bicarbonate solution aerated with 95% (v/v) O₂ and 5% (v/v) CO₂. One end of the specimen was fixed to a shaft and the other end was attached to a pressure transducer (TB-611T; Nihon Kohden, Tokyo) in order to record the tension generated from the specimen on a recorder (SR-6335; Graphtec Corporation, Kanagawa) via an amplifier (AP-621G, Nihon Kohden). After a stabilization period, acetylcholine (100 μ mol/L) and 5-HT (10 μ mol/L) (the concentration inducing a near-maximal response) were repeatedly applied to confirm that consistent responses were observed. The specimen was then exposed to vehicle (Krebs-bicarbonate solution) for 30 min before 5-HT (0.1–30 μ mol/L) was cumulatively applied at a common ratio of approximately three, in order to construct a concentration-response curve. Then, the specimen was washed with Krebs-bicarbonate solution and exposed to test compound for 30 min before the same procedure was repeated, in order to construct other concentration-response curves for 5-HT. A single animal yielded three specimens, and each specimen was used to test one concentration of a particular test compound. Ramosetron, alosetron, and cilansetron were added to final concentrations of 3, 10, and 30; 30, 100, and 300; and 30, 100, and 300 nmol/L, respectively.

Inhibition of the von Bezold-Jarisch (BJ) reflex in rats

Experiments measuring 5-HT-induced bradycardia (the BJ reflex) were performed according to the method of Miyata et al. (18). In this study, rats were under urethane (1.25 g/kg intraperitoneally) (Sigma-Aldrich, Tokyo) anesthesia and artificial ventilation (60 times/min, 3 mL/time) using a polyethylene tube inserted into the trachea. Heart rate and blood pressure were determined via a polyethylene catheter inserted into the left common carotid artery. A heart rate meter (AT-601G, Nihon Kohden) and a blood pressure amplifier (AP-601G, Nihon Kohden) were used to record the heart rate and blood pressure as signals from a pressure transducer (TP-400T, Nihon Kohden) connected to the polyethylene catheter. To measure changes in heart rate, values digitally displayed on the heart rate meter were read before and after administration of 5-HT (30 μ g/kg, intravenously) via a catheter inserted into the left femoral vein.

Each animal received test compounds at one of the following doses (four animals for each dose): ramosetron (0.3, 1, and 3 μ g/kg), alosetron (3, 10, and 30 μ g/kg), and cilansetron (10, 30, and 100 μ g/kg). The test compounds were administered orally 30 min prior to the injection of 5-HT.

Inhibition of restraint stress-induced defecation in rats

In these tests, we used male Wistar rats that had not fasted. Rats were stressed by confinement in a restraining stress cage (KN 468; Natume Seisakusho Co., Ltd., Tokyo) for 1 h (7). The stools were collected 1 h after the initiation of restraint stress and the total weights of stools determined.

The experiment was performed with the following groups (12 rats per group): normal (without restraint stress), 0.5% (w/v) MC, ramosetron (0.3, 1, and 3 μ g/kg), alosetron (3, 10, and 30 μ g/kg), cilansetron (3, 10, and 30 μ g/kg), and loperamide (0.3, 1, and 3 mg/kg). The test compounds were administered orally (5 mL/kg) 1 h before the initiation of restraint stress.

Statistical analyses

All results were statistically analyzed using the Statistical Analysis System version 8.2 (SAS Institute Japan, Ltd., Tokyo). In this study, all data were described after round-off to two significant digits.

After receptor binding assays, the IC₅₀ value of each test compound was calculated using logistic regression from results of the replacement experiment, and the values of K_d and B_{max} were calculated using a Scatchard plot from results of the saturation experiment. Based on IC₅₀ and K_d values, the K_i value of each test compound was calculated according to the equation:

$$K_i = IC_{50} / (1 + [L] / K_d)$$

where [L] is the concentration of radiolabeled ligand.

To obtain a dissociation curve of each test compound from the human 5-HT₃ receptor, the receptor inhibition rates at each time point after the addition of tracer solution were calculated using the equation:

$$\text{Inhibition rate (\%)} = 1 - [(B - N) / (B_0 - N)]$$

where B is bound radioactivity in the presence of test compound, B₀ is total bound radioactivity in the absence of test compound, and N is non-specifically-bound radioactivity.

A dissociation curve was prepared by plotting various inhibition rates, and a dissociation half-life (t_{1/2}) was calculated using linear regression analysis. When a particular inhibition rate was outside the range 5%–95%, this value was excluded, and the t_{1/2} was calculated using values within the acceptance range.

To determine the antagonistic activity of test compounds against 5-HT-induced contraction of isolated guinea-pig colon, the dose ratio was obtained from EC₅₀ values for 5-HT in the presence and absence of an antagonist. A K_B value was determined for each concentration of antagonist according to the following equation:

$$K_B = [\text{antagonist, mol/L}] / (\text{dose ratio} - 1)$$

The pA₂ values were then expressed as negative

Table 1. Affinities of ramosetron, alosetron, and cilansetron for cloned human and rat 5-HT₃ receptors

		Ramosetron	Alosetron	Cilansetron
K _i (nmol/L)	Human	0.091 ± 0.014 (1)	0.29 ± 0.031 (1/3.4)	0.56 ± 0.039 (1/5.6)
	Rat	0.22 ± 0.051 (1)	0.71 ± 0.21 (1/3.3)	0.64 ± 0.15 (1/3.0)

Each value represents the mean ± S.E.M. for three tests. Values in parentheses represent relative affinities compared to ramosetron.

logarithms of the K_B values. In addition, the values of log [dose ratio – 1] were plotted against values for log [antagonist, mol/L], and curvilinear regression lines and slopes were calculated (Schild plots).

The inhibition rates of all doses of each compound against BJ reflux were calculated. From these values, an ED₅₀ value with a 95% confidence limit for each compound was estimated using linear regression analysis.

In the experiment measuring defecation in rats, the mean ± S.E.M. of the total weight of stools in each group was calculated, and Student's *t*-test, or Dunnett's test, was used to make comparisons between normal rats and those treated with 0.5% (w/v) MC, and between rats treated with 0.5% (w/v) MC and test compounds, respectively. To determine inhibitory effects on defecation, an ED₅₀ value for each test compound was estimated from the total weight of stools using linear regression analysis.

Results

Affinities for the 5-HT₃ receptor

In saturation experiments, [³H]-GR65630, a selective radiolabeled 5-HT₃-receptor ligand, specifically bound to membrane preparations of HEK293 cells expressing cloned human 5-HT₃ receptors and COS-1 cells expressing cloned rat 5-HT₃ receptors, with B_{max} values of 45 and 9.1 pmol/mg protein and K_d values of 0.48 and 2.0 nmol/L, respectively. Ramosetron, alosetron, and cilansetron inhibited [³H]-GR65630 binding to human and rat 5-HT₃ receptors in a concentration-dependent manner, demonstrating that these drugs had high affinity for 5-HT₃ receptors. For human 5-HT₃ receptors, the K_i values for ramosetron, alosetron, and cilansetron were 0.091 ± 0.014, 0.29 ± 0.031, and 0.56 ± 0.039 nmol/L, respectively, indicating that the relative receptor affinities of alosetron and cilansetron, compared to ramosetron (1), were 1/3.4 and 1/5.6, respectively (Table 1). For rat 5-HT₃ receptors, the K_i values for ramosetron, alosetron, and cilansetron were

Fig. 1. Time course of changes in the binding of ramosetron, alosetron, and cilansetron to cloned human 5-HT₃ receptors. Each point represents the rate of inhibition by test compound. The test was performed in quadruplicate.

0.22 ± 0.051, 0.71 ± 0.21, and 0.64 ± 0.15 nmol/L, respectively, indicating that the relative receptor affinities of alosetron and cilansetron, compared to ramosetron (1), were 1/3.3 and 1/3.0, respectively (Table 1).

Dissociation from the human 5-HT₃ receptor

The inhibition rates of test compounds binding to the human 5-HT₃ receptor at each time point are plotted in Fig. 1. Ramosetron showed slow dissociation from the 5-HT₃ receptor, with a t_{1/2} value of 560 min, while the t_{1/2} values of alosetron and cilansetron were 180 and 88 min, respectively.

Affinities for 5-HT-receptor subtypes

In all 5-HT-receptor subtypes examined in this study, except the 5-HT₃ receptor, ramosetron at 10 μmol/L did not show an inhibition rate of 50% or more in the binding of the specific radiolabeled ligands (Table 2). On the other hand, alosetron at 10 μmol/L inhibited the binding of [¹²⁵I]-LSD to the human 5-HT_{2B} receptor by 87% and the binding of [³H]-mesulergine to the human 5-HT_{2C} receptor by 72%, with K_i values of 450 ± 32 and

Table 2. Affinities of ramosetron, alosetron, and cilansetron for 5-HT-receptor subtypes

Receptor	Radio-labeled ligand	Inhibition rate at 10 μ mol/L (%)		
		K_i (nmol/L)		
		Ramosetron	Alosetron	Cilansetron
5-HT _{1A} (Human)	[³ H]-8-OH-DPAT	14	32	2.0
		—	—	—
5-HT _{1B} (Rat)	[¹²⁵ I]-iodocyanopindolol	0.0	2.5	25
		—	—	—
5-HT _{2A} (Human)	[³ H]-ketanserin	24	28	0.12
		—	—	—
5-HT _{2B} (Human)	[¹²⁵ I]-LSD	48	87	59
		—	450 $\pm$ 32	2,700 $\pm$ 600
5-HT _{2C} (Human)	[³ H]-mesulergine	39	72	47
		—	2,100 $\pm$ 360	—
5-HT ₄ (Guinea pig)	[³ H]-GR113808	47	37	71
		—	—	1,900 $\pm$ 88
5-HT _{5A} (Human)	[¹²⁵ I]-LSD	27	37	12
		—	—	—
5-HT ₆ (Human)	[¹²⁵ I]-LSD	9.1	18	5.5
		—	—	—
5-HT ₇ (Human)	[¹²⁵ I]-LSD	44	34	16
		—	—	—

Each value represents the mean $\pm$ S.E.M. for three tests. —: not determined, because the inhibition rate was <50%.

2,100 $\pm$ 360 nmol/L, respectively (Table 2). Similarly, cilansetron at 10 μ mol/L showed an inhibition rate of 59% for the binding of [¹²⁵I]-LSD to the human 5-HT_{2B} receptor and 71% for the binding of [³H]-GR113808 to the guinea-pig 5-HT₄ receptor, with K_i values of 2,700 $\pm$ 600 and 1,900 $\pm$ 88 nmol/L, respectively (Table 2).

Affinities for other receptors, ion channels, transporters, and enzymes

When 44 receptors, 5 ion channels, 3 transporters, and 3 enzymes were examined, ramosetron at 10 μ mol/L inhibited the binding of specific radiolabeled ligands by 50% or more in the case of four receptors and one transporter (Table 3). The K_i values for these receptors and transporter, the α_2 -adrenergic receptor (non-selective, rat), the histamine H₂ receptor (rat), the histamine H₃ receptor (rat), the sigma receptor (non-selective, guinea pig) receptors, and the 5-HT transporter (human), were 930 $\pm$ 25, 26 $\pm$ 0.92, 2,400 $\pm$ 600, 4,400 $\pm$ 270, and

1,500 $\pm$ 150 nmol/L, respectively. The affinity ratios for the receptors and the transporter compared to that of the human 5-HT₃ receptor (K_i = 0.091 nmol/L) were 1/11,000, 1/290, 1/27,000, 1/50,000, and 1/17,000, respectively.

Effect on 5-HT-induced contraction of isolated guinea-pig distal colon

In vehicle-treated specimens, 5-HT (0.1 – 10 μ mol/L) contracted the isolated guinea-pig colon in a concentration-dependent manner. Pretreatment with ramosetron (3, 10, or 30 nmol/L), alosetron (30, 100, or 300 nmol/L), or cilansetron (30, 100, or 300 nmol/L) resulted in concentration-dependent rightward shifts of the concentration-response curve for 5-HT (Fig. 2). The values of pA₂ (95% confidence limit) and the slope (95% confidence limit) for ramosetron, alosetron, and cilansetron were 8.6 (8.5 – 9.0) and 0.82 (0.55 – 1.1), 7.5 (7.4 – 7.6) and 1.1 (0.91 – 1.3), and 8.0 (7.9 – 8.3) and 0.91 (0.75 – 1.1), respectively; and the relative

Table 3. Affinities of ramosetron for various receptors and a transporter

Receptor/Transporter	Radio-labeled ligand	K _i (nmol/L)
α_2 -Adrenergic (Non-selective, rat)	[³ H]-RX821002	930 ± 25
Histamine H ₂ (Rat)	[³ H]-cimetidine	26 ± 0.92
Histamine H ₃ (Rat)	[³ H]- <i>N</i> -methyl histamine	2,400 ± 600
Sigma (Non-selective, guinea pig)	[³ H]-1,3-di- <i>o</i> -tolylguanidine	4,400 ± 270
Serotonin transporter (Human)	[³ H]-imipramine	1,500 ± 150

Each value represents the mean ± S.E.M. for three tests.

Fig. 2. Inhibitory effects of ramosetron (A), alosetron (B), and cilansetron (C) on 5-HT-induced contraction of isolated guinea-pig distal colon. Each point with vertical bars represents a mean ± S.E.M. from 4–12 preparations (n = 12, vehicle-treated; n = 4, drug-treated).

Table 4. Potencies of ramosetron, alosetron, and cilansetron on the inhibition of 5-HT-induced contraction in guinea-pig distal colon

	Ramosetron	Alosetron	Cilansetron
pA ₂	8.6 [8.4–9.0]	7.5 [7.4–7.6]	8.0 [7.9–8.2]
Slope	0.82 [0.55–1.1]	1.1 [0.91–1.3]	0.91 [0.76–1.1]
	(1)	(1/13.9)	(1/4.2)

Values in square brackets represent 95% confidence limits from data on 4–12 preparations. (n = 12, vehicle-treated; n = 4, drug-treated). Values in parentheses represent potencies relative to ramosetron.

potencies of alosetron and cilansetron compared to ramosetron (1) were 1/14 and 1/4.2, respectively (Table 4).

Effects on the BJ reflex in rats

Bolus injection of 5-HT (30 µg/kg, intravenously) evoked a transient bradycardia (the BJ reflex). In control rats given 0.5% (w/v) MC, the decrease in heart rate induced by 5-HT was 260 ± 34 beats/min. Oral administration of ramosetron (0.3, 1, or 3 µg/kg), alosetron (3, 10, or 30 µg/kg), or cilansetron (10, 30, or 100 µg/kg) dose-dependently inhibited the BJ reflex, with ED₅₀ (95% confidence limit) values of 1.2 (0.93–1.6), 9.0 (6.9–12), and 40 (25–74) µg/kg, respectively (Table 5). The relative potencies of alosetron and cilansetron compared to ramosetron (1) were 1/7.5 and 1/33, respectively (Table 5).

Inhibition of restraint stress-induced defecation in rats

In normal rats without restraint stress, the total weight of stools after 1 h was 0.34 ± 0.14 g, while restraint stress for 1 h significantly increased this value to 2.7 ± 0.21 g in rats treated with 0.5% (w/v) MC (Fig. 3). Ramosetron (0.3, 1, or 3 µg/kg, orally) significantly inhibited restraint stress-induced defecation in a dose-dependent manner (Fig. 3) with an ED₅₀ (95% confidence limits) value of 0.62 (0.17–1.2) µg/kg (Table 6). Similarly, oral administration of alosetron (3, 10, or 30 µg/kg), cilansetron (3, 10, or 30 µg/kg), or loperamide (0.3, 1, or 3 mg/kg) dose-dependently

Table 5. Potencies of ramosetron, alosetron, and cilansetron in inhibition of the BJ reflex in urethane-anesthetized rats

	Ramosetron	Alosetron	Cilansetron
ED ₅₀ (μg/kg, p.o.)	1.2 [0.93 – 1.6]	9.0 [6.9 – 12]	40 [25 – 74]
	(1)	(1/7.5)	(1/33)

Values in square brackets represent 95% confidence limits from four rats. Values in parentheses represent potencies relative to ramosetron. The ED₅₀ values of all test compounds refer to the hydrochloride forms.

Fig. 3. Effects of ramosetron, alosetron, cilansetron, and loperamide on restraint stress-induced increase in total stool weight in non-fasting rats. Each column with vertical bars represents a mean $\pm$ S.E.M. for 12 animals. ## P <0.01, compared to normal (without restraint stress) rats using Student's t -test. ** P <0.01, compared to control rats treated with 0.5% (w/v) MC using Dunnett's multiple range test. N: normal, C: control. The dose levels of all test compounds refer to their hydrochloride forms.

Table 6. Potencies of ramosetron, alosetron, cilansetron, and loperamide on inhibition of restraint stress-induced defecation in rats

	Ramosetron	Alosetron	Cilansetron	Loperamide
ED ₅₀ (μg/kg, p.o.)	0.62 [0.17 – 1.2]	14 [6.9 – 56]	9.0 [2.9 – 24]	860 [440 – 1,500]
	(1)	(1/22)	(1/15)	(1/1,400)

Values in square brackets represent 95% confidence limits from 12 rats. Values in parentheses represent potencies relative to ramosetron. The ED₅₀ values of all test compounds refer to the hydrochloride forms.

inhibited restraint stress-induced defecation (Fig. 3), and the drugs had ED₅₀ values (95% confidence limits) of 14 (6.9 – 56), 9.0 (2.9 – 24), and 860 (440 – 1,500) μg/kg, respectively (Table 6). The relative potencies of alosetron, cilansetron, and loperamide compared to ramosetron (1) were 1/22, 1/15, and 1/1,400, respectively (Table 6).

Discussion

Although IBS is not fatal, patients with this disease are frequently compelled to limit their social activity

resulting in a decrease in quality of life (19). At present, IBS is treated with spasmolytic agents (anti-cholinergic drugs), anti-diarrheal agents (opioid receptor agonists), lactobacillus preparations, and polymer formulations, but there is insufficient evidence to support their effectiveness (20). Recently, several 5-HT₃-receptor antagonists, such as alosetron (11), cilansetron (12), and ramosetron, have been developed for treatment of IBS-D, and it is anticipated that they will be clinically effective. In this study, we therefore aimed to compare the pharmacological profiles of these drugs.

In receptor-binding experiments, ramosetron inhibited

the binding of [^3H]-GR65630 to cloned human and rat 5-HT₃ receptors in a concentration-dependent manner, with K_i values of 0.091 and 0.22 nmol/L, respectively. Ramosetron at 10 $\mu\text{mol/L}$ did not, however, inhibit ligand binding to other 5-HT-receptor subtypes by >50%. In other experiments using various receptors, transporters, and enzymes, ramosetron showed affinity for the α_2 -adrenergic, histamine H₂, histamine H₃, and sigma receptors, as well as the 5-HT transporter, but the K_i values of ramosetron for these receptors and transporter were much higher than that for the 5-HT₃ receptor. These results indicated that ramosetron is highly selective for the 5-HT₃ receptor. Furthermore, in a functional assay using isolated guinea-pig colon, ramosetron produced a rightward shift of the concentration-response curve for 5-HT, with a pA_2 value of 8.6. Similarly, oral administration of ramosetron dose-dependently suppressed 5-HT-induced transient bradycardia (the BJ reflex), with an ED_{50} value of 1.2 $\mu\text{g/kg}$. Since it is well known that both the 5-HT-induced contraction of colonic smooth muscle (21) and the BJ reflex (22) are mediated by the activation of 5-HT₃ receptors on the enteric nervous system and the nerve endings of vagal afferent neurons, respectively, the results suggest that the inhibitory effects of ramosetron result from antagonism of the 5-HT₃ receptor. In addition, the slope (95% confidence limits) of the curve estimated by Schild plot from the experiments using isolated guinea-pig colon was 0.82 (0.55 – 1.1), indicating that the antagonistic effect of ramosetron on the 5-HT₃ receptor is competitive. Taken together, the data show that ramosetron is a potent and competitive antagonist of the 5-HT₃ receptor.

In all the experiments described above, the affinity and antagonistic activity of ramosetron for the 5-HT₃ receptor was greater than those of other 5-HT₃-receptor antagonists, such as alosetron and cilansetron, indicating that ramosetron, among the 5-HT₃-receptor antagonists developed for IBS-D, possesses the most potent antagonistic activity for the 5-HT₃ receptor. Previously, it has been shown that alosetron possesses affinity and antagonistic activity for the 5-HT₃ receptor, with a K_i value of 0.16 nmol/L (rat brain) and a pA_2 value of 7.7 (guinea-pig ileum) (23, 24). Similarly, cilansetron has been reported to show antagonism for the 5-HT₃ receptor, with values of K_i (rat brain), pA_2 (guinea-pig ileum), and ED_{50} (rat BJ reflex) of 0.19 nmol/L, 7.8, and 26 $\mu\text{g/kg}$ (orally), respectively (25). These data are in close agreement with those of this report. On the other hand, although ramosetron was consistently the most potent drug in all experiments conducted in the present study, the rank order of alosetron and cilansetron activities varied with the experiments. We cannot

exclude the possibility that pharmacokinetic differences between test compounds influence their 5-HT₃-receptor antagonistic activities in vivo, but species and regional differences in the 5-HT₃ receptor may be considerable, as a number of reports have suggested the existence of subtypes of the 5-HT₃ receptor (26, 27). Furthermore, Dubin et al. (28) have reported that the potency of any particular 5-HT₃-receptor antagonist was affected by the subunit composition of the 5-HT₃ receptor, and it is thus highly possible that the affinities of ramosetron, alosetron, and cilansetron for the 5-HT₃ receptor vary depending on the composition of the receptor. We used membrane preparations of cells expressing the homomeric complex of the 5-HT_{3A} for our receptor binding assays. Further experiments are therefore required.

In another series of receptor binding assays, we showed that the dissociation of ramosetron from human 5-HT₃ receptors was extremely slow, with a $t_{1/2}$ value of 560 min. This result is consistent with reports showing that ramosetron had long-lasting inhibitory effects on 5-HT-induced contraction of isolated guinea-pig colon (29) and on the BJ reflex in rats (18, 29). Previously, Ohta et al. (30) reported that the long-lasting binding of ramosetron to the 5-HT₃ receptor was due to the distinctive ability of ramosetron to maintain the active three-dimensional chemical conformation necessary for such binding. On the other hand, the dissociation of alosetron and cilansetron from human 5-HT₃ receptors was relatively fast, with $t_{1/2}$ values of 180 and 88 min, respectively. In clinical trials, alosetron (11) and cilansetron (12) were shown to improve the symptoms of IBS-D with dosing frequencies of two and three times a day, respectively. These results, and literature reports, all suggest that ramosetron may exhibit a longer duration of 5-HT₃-receptor antagonism than does alosetron or cilansetron, and ramosetron may be expected to significantly improve the symptoms of IBS-D at a low dosing frequency (perhaps once a day).

It has been suggested that 5-HT₃ receptors are involved in abnormal defecation induced by stress. This was demonstrated by previous reports showing that a 5-HT₃-receptor agonist stimulated colonic motility (31) and electrolyte secretion (32). Furthermore, stress-induced or stress-related factor-induced abnormal defecation was inhibited by 5-HT₃-receptor antagonists such as ondansetron, granisetron, and ramosetron (7). This study confirms the potent inhibitory effect of ramosetron on restraint stress-induced abnormal defecation in rats; ramosetron potency was greater than those of alosetron and cilansetron, in agreement with results from the receptor binding and functional assays. Similarly, loperamide, an opioid receptor agonist clinically used as an anti-diarrheal agent, also inhibited

restraint stress-induced defecation in rats, in a dose-dependent manner. It has been reported that loperamide inhibited wrap stress-induced defecation in rats at doses of 1–10 mg/kg (33), which is in good agreement with the results of this study. Restraint stress-induced defecation in rats is considered to be useful as an animal model of IBS-D because loperamide is known to improve (decrease) the frequency of defecation and the condition of feces in patients with IBS-D in clinical settings (34). Our work shows that ramosetron, in addition to loperamide, is expected to improve abnormal defecation in patients with IBS-D.

In conclusion, this study has revealed that ramosetron possesses potent, selective, and long-lasting antagonism for the 5-HT₃ receptor and that the drug shows an inhibitory effect on stress-induced defecation in rats. Ramosetron has greater potency than other 5-HT₃-receptor antagonists, such as alosetron and cilansetron, which had been developed for treatment of IBS-D. Our results suggest that ramosetron is a promising therapeutic agent for IBS-D.

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