

Summary

- Oral administration of RQ-0000010 to conscious dogs resulted in marked and long-lasting stimulation of gastric function in a dose-dependent manner.
- No prolongation of QT interval was observed with the treatment of RQ-0000010 at a dose up to 100 mg/kg in dogs. It was considered that RQ-0000010 had over 1,000,000-fold safety margin against cardiovascular safety caused by QT risk.
- Results from the present studies were considered to be clearly supported that RQ-0000010 should have clinical efficacy in the GERD patients without effects on QT interval prolongation.

Background

Gastroesophageal reflux disease (GERD) is one of the most serious acid-related diseases. Successful medications for the relief of symptoms associated with GERD would be worth achieving for continuation of Quality-of-Life (QoL). Rationale for the use of 5-HT₄ receptor agonist for GERD treatment had already been demonstrated in the clinical use of cisapride, which was withdrawn from the market in 2000 for causing Long QT Syndrome. At present, no medicine with gastroprokinetics is available for GERD therapy in the markets. Therefore, potent prokinetic agents with improved cardiac safety have been awaited.

Appendix: Non-Clinical Features of RQ-0000010

Animal Pharmacokinetics and Drug Metabolism

- Oral bioavailability: More than 100% in rats and dogs
- Terminal half-life (t_{1/2β}): ca 13 hours in dogs
- Plasma protein binding: Moderate
- Volume of distribution: Moderate
- Metabolism: No CYPs contribution for elimination from the body
- Clearance route: Biliary and urinary

Safety/Toxicology

- Genotoxicity - Ames (BiolumAmes): Negative with/without rat S-9 activation
In vitro micronucleus: Negative with/without rat S-9 activation
- Photo toxicity: Negative
- Phospholipidosis: Negative
- Cerep: Clean
- General Toxicity: No remarkable findings were noted up to 100 mg/kg in rats and 30 mg/kg in dogs on 7-day repeated dose oral toxicity study.

Acknowledgments

We thank Mitsubishi Chemical Medience Corporation for conducting cardiovascular safety pharmacology study and 7-day repeated dose oral toxicity study in dogs.

Experiment #1: *In vitro* Evaluation

Materials and Methods

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_{2B}, 5-HT_{2C}, 5-HT₄, 5-HT₇, 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 receptors. Radio-labeled ligands were used for each receptor binding assay.

5-HT₄ Agonistic Activity on cAMP Assay

Cells expressing the human 5-HT_{4d} and dog 5-HT_{4h} 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) following the procedure provided from vendor. All experimental steps were done at room temperature.

5-HT₄ Agonistic Activity on Rat Tunica Muscularis Mucosa (TMM)

Male CD IGS rats were purchased from Charles River Laboratories, Japan Inc. (Yokohama, Japan). Rats were euthanized by isoflurane inhalation and a 2 cm segment of intrathoracic esophagus was excised and placed in Krebs' solution. 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., Naunyn-Schmiedeberg's Arch Pharmacol, 343, 439-446, 1991). The strips were suspended in a 10 mL organ bath containing Krebs' solution at 37°C aerated with 95% O₂ and 5% CO₂, under 0.5 g tension. Concentration-response curves were performed after contracting the TMM with carbachol (10 μM).

Results

Table 1 Binding affinities of RQ-0000010 and cisapride for human 5-HT and dopamine receptors.

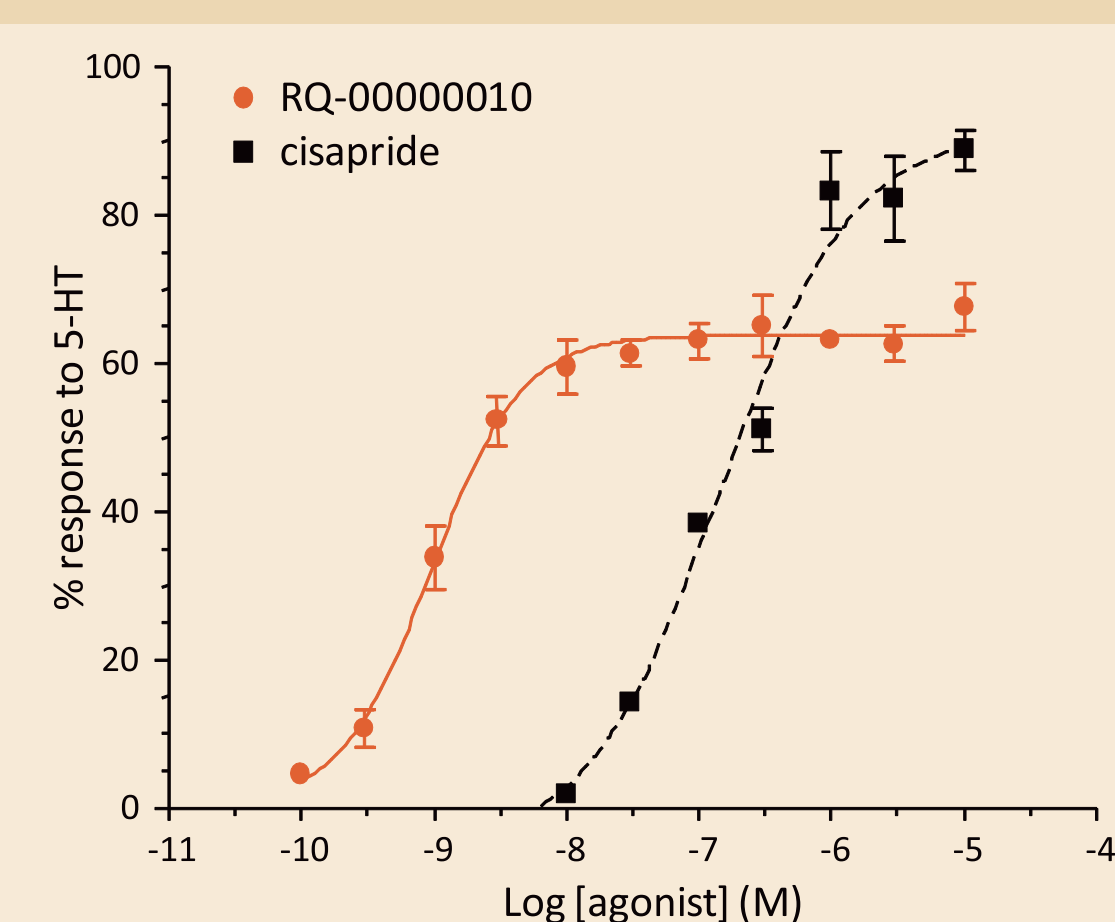
Receptors	Radioligands ^{a)}	K _i (nM)	
		RQ-0000010	cisapride
5-HT _{4d} (human)	[³ H] 5-HT	0.87	158
5-HT _{4h} (dog)	[³ H] 5-HT	1.8	16
5-HT _{1A} (human)	[³ H] 8-OH-DPAT	>5,300	700
5-HT _{1B} (human)	[³ H] 5-HT	>5,600	>5,100
5-HT _{1D} (human)	[³ H] 5-HT	>6,300	680
5-HT _{2A} (human)	[³ H] ketanserin	>5,200	3.1
5-HT _{2B} (human)	[³ H] mesulergine	2,300	not tested
5-HT _{2C} (human)	[³ H] BRL-43694	>4,900	760
5-HT ₇ (human)	[³ H] 5-CT	>4,600	>4,200
D _{2long} (human)	[³ H] spiperone	>2,200	25

a) 5-HT: 5-hydroxytryptamine (serotonin); 8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin; 5-CT: 5-carboxamidotryptamine; BRL-43694: Granisetron

Table 2 Agonistic activities of RQ-0000010 and cisapride in functional assays using HEK293 cells expressing the human 5-HT_{4d} and dog 5-HT_{4h} receptors and rat esophageal TMM tissues.

Function	Parameters	Test Substances	
		RQ-0000010	cisapride
5-HT _{4d} cAMP elevation (human)	EC ₅₀ (nM)	0.74	140
	E _{max} (% of 5-HT)	68	98
5-HT _{4h} cAMP elevation (dog)	EC ₅₀ (nM)	2.3	Not tested
	E _{max} (% of 5-HT)	120	
Tunica Muscularis Mucosae (TMM) (rat)	EC ₅₀ (nM)	2.3	49
	E _{max} (% of 5-HT)	76	57

Figure 1 Effect of RQ-0000010 and cisapride on accumulation on intracellular cAMP in HEK293 cells expressing human recombinant 5-HT_{4d} receptors.



Data for % response to 5-HT (1 μM) were indicated by the mean±S.E.M. (n=4). Results are one representative experiment from three experiments.

Key Findings

- RQ-0000010 showed highly selective and potent affinities to the human and dog 5-HT₄ receptors (Table 1).
- RQ-0000010 is a potent partial agonist against the 5-HT₄ receptors in human and dog cells, and rat TMM (Figure 1, Table 2).

Experiment #2: *In vivo* Evaluation

Materials and Methods

Effect on Gastric Motility

Dogs were implanted extraluminal force transducers (F-12IS, Star Medical, Tokyo, Japan) under anesthesia with isoflurane to the positions as follows: the seromuscular layer of the gastric antrum, (3 cm proximal to the pyloric ring), 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 dogs were placed in a shielded room, and then recording of gut motility in the postprandial state was initiated. The motility was measured with a telemetry system (GTS-800, Star Medical). After confirmation of the incidence of interdigestive migrating complex (IMC) at regular intervals, 10 g/kg of solid meal was given to the dog. Two hours after feeding, RQ-0000010 or vehicle was administered orally. Gastric motility was then recorded for 5 hours. To quantify gastric motility, the areas of the contractions of the gastric antrum were determined by the processing software (Analyze II, Star Medical). The area surrounded by the contraction curve and the baseline for every 1-hour period after administration was calculated, expressed as a percentage of that for the 1-hour period before administration, and used as the post-prandial motor index.

Effect on Gastric Emptying

The test meal was prepared according to the method of Sato et al. (Eur J Pharmacol, 395, 165-172, 2000) with a minor modification. Namely, 10 mL/kg of caloric semi-solid meals thoroughly will be mixed with 10 mg/kg of acetaminophen (APAP). Each dog, which was purchased from Oriental BioService, Inc., was placed in an individual cage after overnight fasting, and water removed. To provide a model of gastroparesis, clonidine (α₂ adrenergic agonist) at a dose of 10 μg/kg was injected subcutaneously 30 minutes before test meal administration. Dogs were orally administered RQ-0000010 or vehicle immediately before the administration of clonidine. The test meal (10 mL/kg) was digested within 2 minutes. Blood samples were collected from cephalic vein at 0, 15, 30, 45, 60, 75 and 90 minutes after test meal administration, and then plasma was separated by centrifugation. Plasma concentrations of APAP and RQ-0000010 were determined by in-house methods using LC/MS/MS.

Results

Figure 2 The effect of orally administered RQ-0000010 on the gastric motility in the postprandial state in conscious dogs.

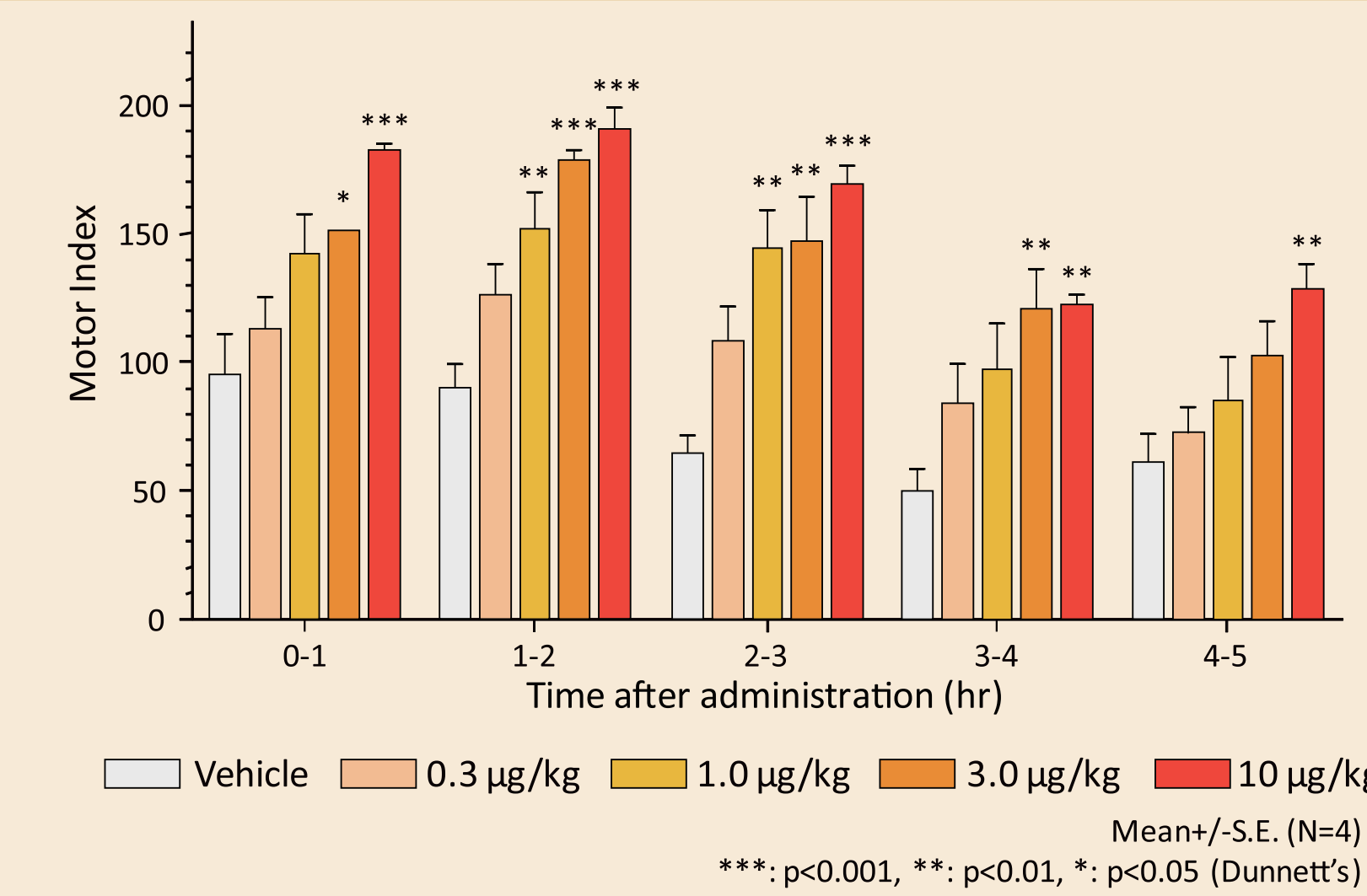
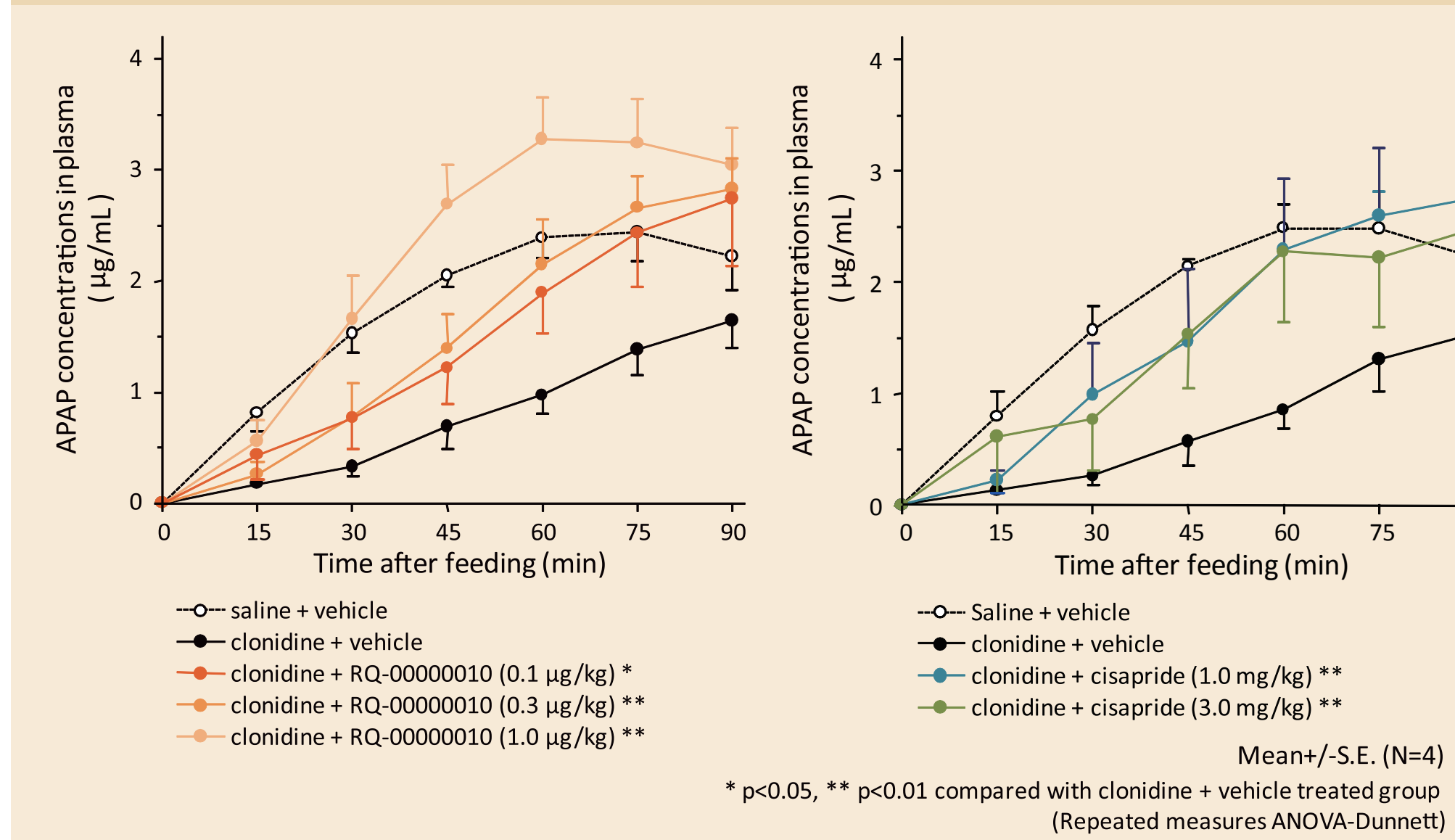


Figure 3 The effects of RQ-0000010 and cisapride on gastric emptying in clonidine-induced gastroparesis in dogs.



Key Findings

- Dose-dependent enhancement in gastric motility with RQ-0000010 was demonstrated in conscious dogs (postprandial) (Figure 2).
- Gastric emptying in dogs was significantly enhanced with the administration of RQ-0000010, under the condition of clonidine-induced gastroparesis and the effects were similar magnitudes with that by cisapride administration (Figure 3).
- Minimum effective dose (MED) of RQ-0000010 to enhance gastric function was confirmed to be 0.1 μg/kg.

Experiment #3: Safety/Toxicology Evaluation

Materials and Methods

HERG (human ether-a-go-go-related gene) Channel Blocking Assay

HEK293 cells expressing the HERG channel were grown in flasks containing suitable medium. 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. 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) back to the holding potential. The voltage pulse was applied to a cell continuously throughout the experiment every 4 s (0.25 Hz) to elicit a large outward current. RQ-0000010 and cisapride were dissolved in DMSO and diluted with external solution [final concentration of DMSO was 0.5% (v/v)] and then applied to the cells. All experiments were carried out at 23±1°C.

Effects on the Cardiovascular System in Dogs (CV safety pharmacology study, 7-day repeated dose toxicity study)

Male Beagle dogs (n=4), previously implanted with radio-telemetry transmitters, were randomly assigned in a 4x4 Partial Latin Square design. Each dog received a single dose of RQ-0000010 by oral gavage at doses of 3, 10 and 30 μg/kg and vehicle (0.5% methylcellulose) with a over 7-day inter-dose washout period between each administration. Dogs were fasted overnight prior to dosing and were fed at approximately 8 hours post-dose. ECG was telemetrically taken from the transmitter through the receiver to an ECG analysis system (hem, Notocord Systems), and recorded from the morning of the day before each administration until 25 hours or more after administration. In addition, the effects on cardiovascular system were also evaluated at the multiple doses of 1, 10, 30 and 100 mg/kg for 7 days on the dose range-finding study in dogs (n=1-2/dose/sex).

Results

Table 3 Effects of RQ-0000010 and cisapride on HERG channels, and its safety margin based on the results from functional assay

	RQ-0000010	cisapride
5-HT ₄ functional EC ₅₀ (E _{max}) ^{a)}	0.74 nM	140 nM (98%)
IHERG assay IC ₅₀	10.5% inhibition @ 30 μM	1.7 nM
Safety margin	> 40,000	< 1

a) Functional assay using HEK293 cells expressing the human 5-HT_{4d} receptor

Table 4 The effect of RQ-0000010 on QT interval in safety pharmacology study in male dogs.

Test Substance	Dose (μg/kg)	N	QT interval (msec) ^{a)}					
			Pre-dose	Time after administration (hr)				
Vehicle ^{b)}	0	4	247±16	237±20	238±7	242±3	229±14	225±29
	3	4	235±12	237±19	232±16	240±11	220±20	232±12
RQ-0000010	10	4	240±11	217±20	228±16	233±12	215±7	229±12
	30	4	226±21	224±9	228±12	233±18	214±11	240±22

This study was conducted in compliance with Good Laboratory Practice Standards for Non-clinical Safety Studies (MHW Ordinance No.21 dated March 26, 1997).

a) ECG was telemetrically taken from the transmitter through the receiver and recorded from the morning of the day before each administration until 25 hours.
b) 0.5% (w/v) methylcellulose

Table 5 The effect of RQ-0000010 on QT interval in 7-day repeated dose oral toxicity study in dogs.

Test Substance	Dose (mg/kg)	N	QT interval (msec) ^{a)}					
			Male		Female			
Vehicle ^{b)}	0	2	Day-2	Day1	Day7	Day-2	Day1	Day7
			183.0	193.5	196.5	196.0	212.0	202.0
RQ-0000010	1	2	193.5	207.0	209.0	180.5	191.5	183.5
	3	2	181.5	195.5	196.5	198.0	199.5	196.5
	30	2	204.0	209.5	212.0	201.5	218.0	215.5
	100	1	188.0	186.0	186.0	198.0	198.0	210.0

a) ECG data was obtained at 1 hour after administration of RQ-0000010 and vehicle.
b) 0.5% (w/v) methylcellulose

Key Findings

- Based on the results from *in vitro* HERG channel assay, RQ-0000010 has over 40,000-fold safety margin for potential risks of cardiovascular adverse effects related to QT prolongation (Table 3).
- GLP safety pharmacology study (CV core battery) showed that RQ-0000010 had no effects on ECG parameters including QT interval up to 24 hours after administration at the doses up to 30 μg/kg (Table 4).
- A 7-day repeated dose oral toxicity study also demonstrated that RQ-0000010 had no adverse effects on QT interval up to 7 days after multiple administration at the doses up to 100 mg/kg (Table 5).
- Based on the estimation using MED, RQ-0000010 has 1,000,000-fold safety margin for the cardiovascular safety risk caused by QT prolongation.