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Tegoprazan, a Novel Potassium-Competitive Acid Blocker to Control Gastric Acid Secretion and Motility

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ABBREVIATIONS:

DMSO, dimethyl sulfoxide; FD, functional dyspepsia; GERD, gastroesophageal reflux disease; H_2, histamine receptor 2; *H. pylori*, *Helicobacter pylori*; HP, Heidenhain Pouch; HTR, mean intra-gastric pH >4 holding time ratio during experimental period; MC, methylcellulose; MMC, migrating motor complex; NAB, nocturnal acid breakthrough; NERD, non-erosive reflux disease; NSAID, non-steroidal anti-inflammatory drug; P-CAB, potassium-competitive acid blocker; PPI, proton pump inhibitor; Tegoprazan, RQ-00000004/CJ-12420/PF-03922155/ (S)-4-((5,7-difluorochroman-4-yl)oxy)-N,N,2-trimethyl-1H-benzo[d]imidazole-6-carboxamide

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ABSTRACT

Tegoprazan, (S)-4-((5,7-difluorochroman-4-yl)oxy)-N,N,2-trimethyl-1H-benzo[d]imidazole-6-carboxamide, a potassium-competitive acid blocker (P-CAB), is a novel potent and highly selective inhibitor of gastric H^+/K^+-ATPase. Tegoprazan inhibited porcine, canine, and human H^+/K^+-ATPases *in vitro* with IC_{50} values ranging 0.29 ~ 0.52 μM while that for canine kidney Na^+/K^+-ATPase was more than 100 μM. A kinetic analysis revealed that tegoprazan inhibited H^+/K^+-ATPase with potassium-competitive manner and the binding was reversible. Oral single administrations of tegoprazan ranging 0.3 ~ 30 mg/kg in dogs were well absorbed into blood stream and distributed in gastric tissue/fluid higher than in plasma. Tegoprazan potently inhibited histamine-induced gastric acid secretion in dogs and a complete inhibition was observed at 1.0 mg/kg starting from 1 hr after administration. Moreover, an oral administration of tegoprazan at 1 and 3 mg/kg reversed the pentagastrin-induced acidified gastric pH to the neutral range. Interestingly, 3 mg/kg tegoprazan immediately evoked a gastric phase III contraction of migrating motor complex (MMC) in pentagastrin-treated dogs and similar effects was observed with the other P-CAB, vonoprazan. Tegoprazan is the novel P-CAB which may provide a new option for the therapy of gastric acid-related and motility-impaired

diseases.

VISUAL ABSTRACT

Not available

Introduction

Acid-related gastrointestinal diseases such as gastroesophageal reflux disease (GERD), non-erosive reflux disease (NERD), gastric ulcer, and non-steroidal anti-inflammatory drugs (NSAIDs)-associated ulcer are the most common diseases among gastrointestinal disorder. Drug therapies starting from histamine receptor 2 (H_2) blockers and following proton pump inhibitors (PPIs) demonstrated the efficacy of the inhibition of gastric acid secretion for the treatment of acid-related diseases and those new drugs dramatically improved the quality of life of the patients. However, a level of satisfaction of the therapy with the currently available drugs is still inadequate. For example, the control of heartburn and esophageal reflux symptoms during night time are difficult in the current PPI therapy, and the symptoms of patients during initial 3 days after PPI therapy are not controlled (Strand et al., 2017; Chey et al., 2010; Fass et al, 2009). The common properties of the currently available PPIs, e.g. PPIs require chemical transformation to active form and simultaneous activation of H^+/K^+-ATPase on parietal cell membrane for the inhibition of acid secretion, bind to the target molecule irreversibly, and eliminate from plasma rapidly, often cause an insufficient gastric pH control which may result in therapeutic failure (Hunt, 2012; Inatomi et al, 2016).

A new class of drug vonoprazan, a potassium-competitive acid blocker (P-CAB) which inhibits gastric H^+/K^+-ATPase through a different mechanism with PPI, was recently approved in Japan and it demonstrated potent therapeutic efficacies on reflux esophagitis, gastric and duodenal ulcer, low-dose-aspirin/NSAIDs-mediated ulcer, and *Helicobacter pylori* (*H. pylori*) eradication. The efficacy of vonoprazan in patients is reported to be superior to those of PPIs in general. The pharmacological advantages of vonoprazan in human are supposed to be result from better control of gastric acid secretion reaching the neutral pH range (pH >7) and sustainable drug distribution in gastric tissue (Miwa et al., 2017). In this manuscript, we characterized tegoprazan, a novel P-CAB currently in the late stage of clinical development in Asian countries, and demonstrated its potent and long-lasting pharmacological efficacies in animal acid-related disease models.

A migrating motor complex (MMC) is well characterized by an appearance of gastrointestinal contractions in the inter-digestive state in dogs and humans. The MMC consists of four phases: phase I (quiescent period), phase II (period of intermittent and irregular low-amplitude contractions) and phase III (period of short burst of regular high-amplitude contractions) and phase IV (transition period back to the phase I). The physiological importance of gastric MMC pertains to the mechanical

and chemical cleansing of the empty stomach in preparation for the next meal. Phase III contraction of MMC is a series of gastrointestinal pro-kinetic contraction with slow wave frequency and high amplitude, and is observed repeatedly with 60 ~ 90 min interval in the empty stomach (Takahashi, 2013). Since several evidences indicated the relationship between gastric acid inhibition and a disorder of phase III MMC, we evaluated the pharmacological effects of tegoprazan to evoke gastric phase III MMC contraction in pentagastrin-treated dog model (Parkman et al, 1998).

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Materials and Methods

Chemicals

Tegoprazan, vonoprazan, omeprazole, and SCH28080 were synthesized by RaQualia Pharma Inc (Hanazawa et al, 2007; Kajino et al, 2006; Gustavsson et al, 1997; Kaminski et al, 1985). All the reagents and solvents used were analytical grade or higher.

Ethics Approvals and Animals

All procedures were carried out with the approval of the Animal Ethics Committee at the institutes in which the study conducted, RaQualia Pharma Inc. (Aichi, Japan) or Pfizer Nagoya Laboratories (Aichi, Japan), according to the Laboratory Animal Welfare guidelines. Porcine stomachs were purchased from a local abattoir. Beagle dogs were purchased from Oriental BioService Inc. (Kyoto, Japan), Marshall BioResources Japan (Tsukuba, Japan) or Beijing Marshall Biotechnology Co., Ltd (Beijing, China).

Preparation of Porcine and Canine Gastric H^+/K^+-ATPase Vehicles

Porcine and canine H^+/K^+-ATPase vehicles were prepared as previously reported with some modifications (Mori et al., 2009). Briefly, a fresh porcine stomach was washed with saline on ice. The fundic mucosa was scraped off from the underlying muscular layer, minced and homogenized in a 250 mM sucrose solution. The

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homogenate was filtered with gauze, and then centrifuged at 20,000×g for 30 minutes at 4°C. The supernatant was centrifuged at 115,000×g for 30 minutes at 4°C. The pellet was suspended in a 250 mM sucrose solution and separated by a differential and zonal density gradient centrifugation, 250 mM sucrose (upper layer) and 7% (w/v) Ficoll in 250 mM sucrose (lower layer), at 130,000×g for 60 minutes at 4°C with a vertical rotor. The fraction above the Ficoll interface was collected and diluted ten times with water (ion-tight vehicle) and permeabilized by freeze-dry processing (ion-leaky vesicle) and stored at -80°C. Before use, the freeze-dried vesicles were reconstituted with water up to the original volume. The protein concentration of reconstituted vesicles was determined using the BCA protein assay kit protocol (Pierce, USA).

Fresh canine stomachs were washed with saline on ice. The fundic mucosa was scraped, minced and homogenized with a homogenizer in a solution containing 1 mM EGTA, 250 mM sucrose and 5 mM Tris (pH 7.4 at 4°C). The homogenate was filtered with gauze, and then centrifuged at 20,000×g for 30 minutes at 4°C, and then the supernatant was centrifuged at 115,000×g for 30 minutes at 4°C. The pellet was suspended in a 250 mM sucrose solution and separated by the density gradient centrifugation (the same construction to that used for porcine preparation) at

132,000×g for 90 minutes at 4°C with a swing rotor. The following procedures were the same as that for porcine stomach.

Preparation of Human Recombinant H^+/K^+-ATPase Vehicles

Human recombinant H^+/K^+-ATPase was prepared as described previously with some modifications (Mori et al., 2009). Briefly, HEK293 cells stably expressing the human gastric H^+/K^+-ATPase were prepared by Pfizer Inc. The cells suspended in the DMEM containing 10% heat inactivated FBS, 0.5 mg/ml G418, 0.1 mg/ml zeocin, 100 unit/ml penicillin and 100 µg/ml streptomycin were seeded in T-225 cell culture flask. After 11 days culture in humidified incubator at 37°C with 5% CO_2, the cells were harvested in 1 mM EDTA/PBS without Mg^{2+}/Ca^{2+}. The cells were centrifuged at 1,000 rpm for 5 minutes at 4°C. The packed cells were suspended in a buffer containing 0.5 mM $MgSO_4$, protease inhibitors (Roche Complete) and 50 mM Tris-HCl (pH 7.4), and then homogenized with a Polytron homogenizer for 40 seconds. The homogenate was centrifuged at 1,000 rpm for 5 minutes at 4°C. The supernatant was re-centrifuged at 40,000×g for 30 minutes at 4°C. The pellet was suspended in a 250 mM sucrose solution with a Polytron homogenizer and was aliquoted and stored at –80°C until use. The protein concentration of the membrane fraction was determined using the BCA protein assay kit protocol (Pierce, USA).

Measurement of H^+/K^+-ATPase Activities

The H^+/K^+-ATPase activates were determined as described previously with some modifications (Mori et al., 2009; Keeling et al., 1988). The porcine and canine gastric H^+/K^+-ATPase activates were measured in a 60 µL reaction mixture containing 0.3 µg for porcine or 0.7 µg for canine freeze-dried vesicles (ion-leaky vesicles), test compounds, 5 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2ATP and 40 mM Bis-tris (pH 6.4) at 37°C in a 96-well polystyrene plate. For 0 and 100% inhibition controls, the enzyme reaction was performed in the presence of 1% dimethyl sulfoxide (DMSO) and 100 µM SCH28080, respectively. The enzyme reactions using porcine ion-tight vesicles were assayed in a 60 µL reaction mixture containing 2 µg of vehicles, test compounds, 150 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2ATP, 17 µM valinomycin (Sigma), and 5 mM Tris (pH 7.4) at 37°C. The reaction was started by the addition of Na_2ATP and the mixture was incubated at 37°C for 30 min, and then 30 µl of 10% SDS containing antifoam A was added to stop the reaction. Then 200 µL of the colorimetric reagent to analyze inorganic phosphate consisting from four parts of 10% L-ascorbic acid (pH 5) and one part of 35 mM ammonium molybdate in 15 mM zinc acetate (pH 5) was added into each well. Following incubation at 37°C, the optical density was measured at 750 nm with ARVOsx (PerkinElmer). The inorganic phosphate solution prepared from

KH_2PO_4 and K_2HPO_4 solutions was used as a standard of the inorganic phosphate. Test compounds were dissolved in DMSO at 10 mM and then diluted in the reaction mixture to give appropriate final concentrations. The human recombinant H^+/K^+-ATPase activity was measured in a 60 µl enzyme reaction mixture containing 10 µg of H^+/K^+-ATPase vehicle, the test compound, 10 µM oligomycin, 100 nM bafilomycin A1, 10 µM thapsigargin, 10 µM ouabain, 5 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2ATP and 40 mM Bis-tris (pH 6.4 at 37°C) in a 96-well plate. The reaction mixture was incubated for 50 minutes at 37°C after addition of Na_2ATP, and 30 µl of 10% SDS containing antifoam A was dispensed to stop the reaction. The colorimetric analysis was performed in the same procedure to porcine assay.

Kinetic Analysis of H^+/K^+-ATPase Inhibition

The enzyme kinetic study was performed based on the method of porcine ion-leaky assay described above except for the amount of vehicle (1 µg), and tested with various concentration of potassium (final concentrations of 2.0, 2.5, 3.5, 5.0, and 10 mM KCl) and tegoprazan (final concentrations of 0.15, 0.30, 0.45, and 0.60 µM).

Measurement of Canine Kidney Na^+/K^+-ATPase Activity

Canine kidney Na^+/K^+-ATPase was purchased from Sigma (A-0142). The enzyme was diluted to 7 mg/mL with 250 mM sucrose solution. The canine kidney

Na^+/K^+-ATPase activity was measured in a 60 µL enzyme reaction mixture containing 11µg of protein, test compound, 100 mM NaCl, 2 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2ATP, 40 mM Tris (pH 7.4 at 37°C) in a 96-well polystyrene plate. For 0 and 100% inhibition controls, the enzyme reaction was performed in the presence of 1% DMSO and 100 µM ouabain, respectively. The reaction was started by the addition of Na_2ATP and the mixture was incubated for 30 minutes at 37°C, and 30 µl of 10% SDS containing antifoam A was added to stop the reaction. The colorimetric analysis was performed in the same method to porcine assay.

Data Analysis

All the H^+/K^+- ATPase and Na^+/K^+-ATPase assays were conducted in triplicate. The results of three independent experiments were used for analysis. The nonlinear curve fitting and IC_{50} determination and the kinetic study using Lineweaver-Burk plot were analyzed using GraphPad Prism version 5 (GraphPad Software Inc., CA, USA). The values are shown as mean ± S.E.M.

Measurement of Plasma and Gastric Juice Concentration of Tegoprazan in Dogs

Male beagle dogs at 8 ~ 11 months old were fasted overnight prior to dosing and dosed tegoprazan with 0.3, 3, and 30 mg/kg PO (n=2) and blood were collected at 0.25, 0.5, 1, 2, 4, 8, and 24 hrs post dosing. Male Heidenhain Pouch (HP) dogs were orally

dosed tegoprazan with 1 or 3 mg/kg, and blood and gastric juice were collected at 5 or 16 hrs after the drug dose. Plasma and gastric concentrations of tegoprazan were quantified using LC-MS/MS (API4000 Triple Quadrupole mass spectrometer, Applied Biosystems, CA, USA) and calculated using Analyst Program ver. 1.4.1 (Applied Biosystems, USA). Pharmacokinetic parameters were determined with non-compartmental analysis using WinNonlin ver 5.2.1 (Pharsight Corporation, NC, USA).

Inhibition of Binding/function against Pharmacologically Relevant Molecules

Inhibitions of binding or functional activity against pharmacologically relevant molecules, receptors, ion channels, transporters, and enzymes, were tested in the presence of 10 μM tegoprazan. Regarding the Na^+/K^+-ATPase, tegoprazan was tested at 30 μM. All the assays were conducted by Eurofins Cerep (France) under their validated assay methods in the presence of standard inhibitors as an assay control (http://www.cerep.fr/cerep/users/pages/catalog/assay/catalog.asp).

Measurement of Gastric Acid Secretion in HP Dog Model

A gastric pouch was constructed according to a method introduced by Heidenhain (Heidenhain, 1879). Briefly, male beagle dogs (7 ~ 12 kg body weight) were anesthetized with midazolam (0.2 mg/kg, i.m.) and medetomidine (0.05 mg/kg, i.m.)

and isoflurane inhalation. The abdominal cavity was opened after the injection of atropine sulfate (0.5 mg/mL, 1 mL, i.m.) and infusion of Lactec® D (100 mL/hr). After exposing the stomach in the surgical field, a portion of the greater curvature opposite the splenic hilum was converted into a pouch with an adequate blood supply from the intact gastroepiploic artery. The main body of the stomach was reconstituted, while the pouch drained into an implanted metal cannula. After closing the pouch, the cannula was brought out of the abdominal cavity through the left lateral abdominal wall. Animals were allowed to recover from surgery for at least at last 3 weeks. The experiment was started after overnight fasting with free access to water. Acid secretion was stimulated by continuous intravenous infusion of histamine (Sigma, 80 μg/mg/hr) and maintained throughout the experimental periods. Gastric juice samples were collected by gravity drainage every 15 min throughout the experiment. At 90 min after initiation of the histamine infusion, tegoprazan, omeprazole or vehicle (0.5 % methylcellulose (MC), Wako, Japan) was administrated orally. In the 5-day repeated dosing study, test drug or vehicle was administered orally for 5 days and its inhibitory effect on histamine-stimulated acid secretion was examined at the treatment day 1 and day 5. The collected gastric juice samples were centrifuged and acidity in the supernatant was determined using an automatic titrator (AUT-501, TOA/DKK, Tokyo,

Japan). Gastric acid secretion every 15 min interval (acidity x volume of the supernatant) was expressed as milliequivalent per 15 min (mEq/15 min). All results were calculated by MS EXCEL software (Microsoft, USA).

Measurement of Gastric pH in Dogs

Gastrostomy was conducted as follows. The dogs are anesthetized with midazolam (0.2 mg/kg, intramuscular) and medetomidine (0.05 mg/kg, intramuscular) and isoflurane inhalation. The abdominal cavity was opened after the injection of atropine sulfate (0.5 mg/mL, 1 mL, intramuscular) and infusion of Lactec® D (100 mL/hr). A gastrostomy tube (D12-C, 6 cm, Natsume Seisakusho Co. Ltd., Japan) was connected to the center of body of stomach and closed. Gastrostomy tube was taken out from the abdominal cavity throughout the left lateral abdominal wall and closed. The experiments were started at least one month after the surgery.

After overnight fasting, the dogs were placed in suspended-type dog slings and attached a dip-type reference microelectrode (MI-402, Microelectrode Inc.) in the back. An esophageal pH microelectrode (MI-508, Microelectrode Inc.) was introduced into the gastrostomy tube and fixed at 10 mm above gastric lumen. Both the microelectrodes were connected to pH meter (F52, Horiba, Japan) and measured gastric pH values. During the pH measurement, pentagastrin (Sigma, 6 μg/kg/hr,

subcutaneous infusion) was injected continuously. Test drug or vehicle was administered through oral or intravenous 1.5 hrs after starting of pentagastrin injection and pH was measured more than 5 hrs after drug administration. In the 5-day consecutive administration experiment, drugs were administered before feeding on Day 1 ~ Day 4 and the procedures above were done on Day 5. The gastric pH values were gathered 5 or 6 second interval and the median of each 15 min were used for the analysis. The graph shows mean of the median from 4 dogs.

Measurement of Gastric Antral Motility in Conscious Dogs

The gastric antral motility was determined as described previously (Mikami et al., 2008). Briefly, healthy male beagles weighing 9 to 15 kg, were anesthetized with isoflurane, and the abdominal cavity was opened under aseptic conditions. Extraluminal force transducers (IS-12S; Star Medical, Tokyo, Japan) were sutured onto the seromuscular layer of the gastric antrum 3 cm proximal to the pyloric ring, according to the method of Ito (Ito et al., 1977). The transducer's lead wires were taken out of the abdominal cavity through a skin incision between the scapulae. After surgery, the dogs were placed in protective jackets and housed in individual cages. Gastric motility was recorded starting at least 2 weeks after surgery. After an overnight fast, the dogs were placed in a shielded room, and gastric motility was

recorded in the fasted state. Gastric motility was measured with a telemetry system (GTS-800; Star Medical, Tokyo, Japan), and data were acquired on a personal computer with acquisition software (Eight Star; Star Medical, Tokyo, Japan). After confirming the incidence of an interdigestive migrating complex (IMC, a typical motility pattern of the upper gastrointestinal tract in the fasted state) at regular intervals, test drugs or vehicle (0.1% MC) was administered orally, and gastric motility was recorded for 8 hrs. To measure the gastric motility quantitatively, motor indexes that represent areas of contractions were calculated. The signals from the force transducer were acquired on a personal computer and analyzed by processing software (Analyze II; Star Medical, Tokyo, Japan).

Results

Tegoprazan Inhibits Porcine, Canine, and Human H^+/K^+-ATPase Activity

The inhibitory effects of tegoprazan on porcine, canine stomach H^+/K^+-ATPase, and on recombinant human H^+/K^+-ATPase expressed in HEK293 cells were tested. The H^+/K^+-ATPase vehicle preparation method initially provides an ion-tight vehicle. Since the potassium binding site of the H^+/K^+-ATPase exists inside of the vehicle in this preparation, potassium ion at outside of the vehicle cannot access to the ATPase without

a presence of ionophore. The ion-tight vehicles were then processed with freeze-dry procedure to destroy the vehicle structure to provide the ion-leaky vehicle preparation. In the ion-leaky vehicle, potassium can freely access the potassium-binding site in the assay solution (Reenstra et al., 1990). We determined the inhibitory activities of tegoprazan on the H^+/K^+-ATPase using the ion-leaky vehicles. Inhibitions of H^+/K^+-ATPase purified from porcine, canine stomach and human recombinant by tegoprazan were dose-dependent and the IC_{50} values were 0.47, 0.29, and 0.52 μM, respectively (Table 1, Fig. 2). These results suggested that there is no species-specific inhibition of H^+/K^+-ATPase by tegoprazan among tested. The inhibition of ATPase activity by tegoprazan was also tested using the porcine ion-tight vesicle preparations. IC_{50} value of tegoprazan in porcine ion-tight vesicle was 0.13 μM which was slightly more potent than that for porcine ion-leaky vesicle (Table 1).

Tegoprazan is the Highly Selective Inhibitor of H^+/K^+-ATPase

The H^+/K^+-ATPase is a member of P-type ATPase family. In this family, Na^+/K^+-ATPase, expressed in renal tubular cells and taking an important role in ion transport at kidney, has similar molecular structure to H^+/K^+-ATPase. To identify the selectivity of tegoprazan in the family, inhibitory effect of tegoprazan on canine kidney Na^+/K^+-ATPase was determined. Tegoprazan inhibited Na^+/K^+-ATPase less than 30%

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at a concentration of 100 μM. The results indicate tegoprazan show more than 340-fold selective inhibition of canine H^+/K^+-ATPase over canine Na^+/K^+-ATPase (Table 1). The selectivity of tegoprazan was further determined against various pharmacologically relevant molecules *in vitro*. Inhibition of specific-ligand bindings or functional activities by 10 μM tegoprazan on receptors, enzymes, ion channels, and transporters were measured. 10 μM tegoprazan did not inhibit the binding or functional activities of the molecules listed in Table 2 more than 50% except for rat melatonin receptor ML_2 (MT_3). Tegoprazan at 10 μM inhibited 81% of the binding of melatonin to ML_2 receptor and the IC_{50} value was 1.2 μM.

Tegoprazan Inhibits Gastric H^+/K^+-ATPase in a Potassium-competitive and Reversible Manner

To study the kinetics of the inhibition of H^+/K^+-ATPase by tegoprazan, experiments with various concentrations of potassium and tegoprazan were conducted and analyzed using Lineweaver-Burk plot. The inhibition of H^+/K^+-ATPase by tegoprazan was potassium-competitive manner as shown in Fig. 3A. This potassium-competitive typical kinetic result indicates that the binding of tegoprazan to H^+/K^+-ATPase is reversible. The reversibility was also confirmed by the other experiment which compares the inhibitory potencies of tegoprazan between before and after the dilution of

tegoprazan concentration in the H^+/K^+-ATPase enzyme reaction. Tegoprazan at 3 μM inhibited 86% of H^+/K^+-ATPase activity, whereas the inhibition was decreased to 34% after the dilution of tegoprazan concentration to 0.15 μM. The results suggested that tegoprazan once bound to H^+/K^+-ATPase at higher concentration was eliminated by decreasing tegoprazan concentration in the reaction mixture (Fig. 3B).

Tegoprazan Highly Distributes in Gastric Juice than in Plasma in Dogs

To determine the oral exposure of tegoprazan in dogs, plasma pharmacokinetic profile was tested. Single oral dosing of tegoprazan at 0.3, 3, and 30 mg/kg, pharmacologically effective dose range, to Beagle dogs were well absorbed into the systemic circulation with dose-proportional manner (Fig. 4A). The C_{max} and $AUC_{0\text{-inf}}$ were 2,490 ng/mL and 12,731 ng· hr/mL, respectively, at oral 3 mg/kg dosing. The elimination of tegoprazan from the blood flow was relatively rapid and the plasma half-life were in the range of 3.3~3.5 hrs at the doses tested (Table 3). To understand the exposure of tegoprazan in the targeted disease organ, tegoprazan concentration in gastric juice after oral administration was then determined and compared to that in plasma. Tegoprazan was dosed to HP dogs (N=3) at 1 or 3 mg/kg orally and gastric juice and blood samples were collected at 5 or 16 hrs after tegoprazan administration, respectively, and the drug concentrations were analyzed. The mean tegoprazan

concentrations in gastric juice were higher than those in plasma at 5 hrs after oral 1 mg/kg dosing and 16 hrs after 3 mg/kg dosing (Fig. 4B).

Tegoprazan Inhibits Gastric Acid Secretion in HP Dog Model

To identify the therapeutic efficacy of tegoprazan in animal disease model, effects of single administration of tegoprazan on a gastric acid secretion were determined in the HP dogs. The gastric acid secretion was increased by intravenous infusion of histamine treatment (80 μg/kg/hr) to a plateau level within 90 min and this effect was maintained for more than 5 hrs. Oral single administration of tegoprazan doses at 0.1, 0.3 and 1.0 mg/kg dose-dependently inhibited the histamine-induced gastric acid secretion and a complete inhibition was observed at 1 mg/kg within 1 hr after tegoprazan dosing. Tegoprazan at 1 mg/kg in dogs is a dose corresponding to the human clinical dose used in a Phase II study, 50 or 100 mg once a day (Clinical Trials, NCT03006874); 1 or 2 mg/kg based on a calculation with the human body weight as 50 kg. In this experiment, the efficacies of all the doses of tegoprazan were maintained for more than 5 hrs after tegoprazan administration (Fig. 5A). To determine further long-term efficacy of tegoprazan on the control of gastric acid secretion, the histamine infusion was started 14.5 hrs after the tegoprazan administration and the change of gastric acid secretion was monitored until 21 hrs. The oral tegoprazan at 3 mg/kg

inhibited histamine-induced gastric acid secretion for more than 21 hrs after dosing (Fig. 5B). To test the rapidness of pharmacological onset of tegoprazan in comparison with PPIs, the efficacies of tegoprazan and omeprazole on gastric acid secretion were measured on day 1 and on day 5 after consecutive administration. The oral tegoprazan at 0.3 mg/kg inhibited gastric acid secretion potently on day 1 and the inhibitory potency was similar to that observed on day 5 (Fig. 5C). Inhibition of gastric acid secretion by omeprazole at 0.6 mg/kg, a dose corresponding to the human clinical dose (20 or 40 mg once a day), was weak to moderate on day 1, however, that on day 5 was almost complete (Fig. 5D). The experiments demonstrated that tegoprazan show maximum inhibition of acid secretion from the day of initial treatment while the inhibition by omeprazole on day1 is weak or moderate in dogs.

Tegoprazan Reverses Pentagastrin-induced Gastric pH Decrease in Dogs

Therapeutic efficacy of tegoprazan was also determined in terms of the intra-gastric pH control in dogs. After overnight fasting, the subcutaneous pentagastrin infusion with 6 μg/kg/hr to the dogs resulted in a stable gastric pH approximately 2. Single oral administrations of 0.3, 1, and 3 mg/kg tegoprazan at 1.5 hrs after the initiation of pentagastrin treatment demonstrated dose-dependent gastric pH escalation. Tegoprazan at 1 mg/kg increased gastric pH more than 7 at maximum and a mean

intragastric pH >4 holding time ratio during experimental period (HTR) was 54%. Three (3) mg/kg of tegoprazan achieved stable gastric pH to neutral range within 1 hr and the effect was sustained throughout the period of experiment and the HTR was 89% (Fig. 6A). An efficacy of vonoprazan on the gastric pH control was determined and compared to that of tegoprazan in this model. A single oral vonoprazan at 0.4 mg/kg, a dose corresponding to the human clinical dose (10 ~ 40 mg a day), showed moderate and sustained increase of gastric pH ranging 4 ~ 5 and the HTR was 33%. The complete pH control corresponding to tegoprazan 3 mg/kg was observed with 1 mg/kg vonoprazan (Fig. 6B). These experiments demonstrated that the efficacy of P-CABs, tegoprazan and vonoprazan, on the gastric pH was potent to achieve neutral range.

Tegoprazan Activates Gastrointestinal Motility in Dogs

In addition to the control of gastric pH, an ability to control the gastrointestinal motility by the drugs is the other index for the therapy of some types of gastrointestinal diseases. Effects of tegoprazan on the gastric motilities were tested using telemetry system in dogs. The dog gastric antrum after overnight fasting caused a phase III MMC contraction with 1~2 hrs interval (Fig. 7A upper). A subcutaneous continuous injection of pentagastrin (6 μg/kg/hr, 1 mL/hr) which stimulate gastric acid secretion completely disappeared the phase III MMC signals and started an irregular and

low-height waves similar to the contractile pattern after feeding (Fig. 7A middle). Oral administration of tegoprazan at 3 mg/kg to the dog after the pentagastrin-treatment suppressed the pentagastrin-induced signals and resumed the phase III MMC signals (Fig. 7A bottom). A similar effect on the gastric phase III MMC contraction was also observed with oral vonoprazan at 1 mg/kg, the dose which increased gastric pH to neutral range in dogs (Fig. 7B).

Discussion

In vitro pharmacological studies demonstrated that tegoprazan has potent inhibitory activities of porcine, canine, and human H^+/K^+-ATPases in the ion-leaky vehicle preparation with the IC_{50} values ranging 0.29 ~ 0.52 μM and there was no species-selective difference of inhibition among species tested. The IC_{50} value of tegoprazan in porcine H^+/K^+-ATPase in the ion-tight vehicle preparation was 0.13 μM. The IC_{50} value of the canine kidney Na^+/K^+-ATPase, a highly homologous molecule to H^+/K^+-ATPase, by tegoprazan was more than 100 μM, and the results suggest that tegoprazan shows more than 340-fold selective inhibition to H^+/K^+-ATPase over Na^+/K^+-ATPase in dogs. The study against 115 pharmacologically-relevant molecules further revealed the excellent selectivity profile of tegoprazan against wide range of

molecules except for ML_2 (MT_3) receptor. Inhibition of H^+/K^+-ATPase by tegoprazan was potassium-competitive and reversible manner and these profiles were the same that reported for vonoprazan (Miwa et al., 2017; Kim et al., 2010). This result indicates that tegoprazan has a potential to show pharmacological profiles similar to those of vonoprazan in human.

The pharmacokinetic evaluation of single oral dose of tegoprazan in dogs demonstrated that efficient and dose-proportional absorption into the blood flow. However, the plasma half-life of the tegoprazan, 3.3 ~ 3.5 hrs, was not long enough to maintain the protein-unbounded plasma drug concentration above the IC_{50} value in H^+/K^+-ATPase *in vitro* assay at ~20 hrs after the oral dosing. Then, we analyzed the concentration of tegoprazan in gastric juice, the target tissue/fluid. The mean concentration of tegoprazan in gastric juice at 16 hrs after the oral 3 mg/kg administration was 603 ng/mL and it was approximately 6-fold higher than that in plasma. A protein-unbounded tegoprazan concentration in this gastric juice was calculated to be 130 nM (a protein binding ratio of tegoprazan in dog plasma was used for the calculation), and it was in the range of the *in vitro* IC_{50} values of H^+/K^+-ATPase tested in canine ion-leaky preparation (290 nM) as well as in porcine ion-tight preparation (130 nM) which is supposed to maintain *in vivo* environment of

H^+/K^+-ATPase better than that in ion-leaky preparation (Reenstra et al., 1990). The results suggested that the sustainable existence of tegoprazan in the gastric tissue/fluid enable the long-term gastric acid control in the dogs.

The oral tegoprazan administration at the doses corresponding to human use potently suppressed gastric acid secretion from the initial day of the treatment in dog model. The potency of the inhibition of acid secretion on Day 1 was almost the same to that on Day 5 and the results indicates tegoprazan show maximal efficacy from the initial day of the treatment. In contrast, the inhibition of gastric acid secretion by oral omeprazole also at the dose corresponding to human use was weak to moderate on day 1 while that after daily 5 days administration was maximum. In the clinical use of omeprazole and other PPIs, the maximum efficacy on the acid suppression is generally obtained 3 ~ 5 days after the initiation of the drug dosing (Strand et al, 2017). Since the evidence of omeprazole observed in this dog model precisely reflects the delayed onset generally observed in clinical environment, the results observed for tegoprazan in the same model strongly suggested the opportunity of the potent inhibition of gastric acid secretion by tegoprazan from the initial day of the therapy in human use.

We demonstrated that the single dosing of tegoprazan at 1 and 3 mg/kg, the doses corresponding to human use, achieved maximum gastric pH more than 7, and the single

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3 mg/kg dosing of tegoprazan maintained gastric pH more than 6.5 for 5 hrs after the drug administration. In another study, single 3 mg/kg tegoprazan continuously inhibited gastric acid secretion until 21 hrs after the administration. Besides, a similar potent and stable control of gastric pH by 1 mg/kg vonoprazan was reported in contrast to a limited potency and short term efficacy of 10 mg/kg lansoprazole, a clinically available PPI, in a rat model (Hori et al., 2010). Above results suggest that P-CABs may generally have the efficacy to control gastric pH reaching neutral range for almost a day and the efficacy exceeds those of PPIs. These properties of tegoprazan, potent and long-lasting acid inhibitor, may be able to contribute to the better satisfaction e.g. in PPIs-refractory patients.

There are several evidences that show the increase of gastric pH with inhibitors of acid secretion stimulate the phase III MMC contraction, however, the results are controversial (Parkman et al, 1998, Gielkens et al, 1998). To identify whether the above evidence could be observed with P-CABs, we determined tegoprazan, as one of the most potent and quick inhibitor of acid secretion, in our dog model. We demonstrated that tegoprazan potently and quickly recovered the impaired gastric phase III MMC in the dog model. The similar effect was also identified with another P-CAB, vonoprazan. Since the stable and reproducible pharmacological effects on phase III

contraction were observed by tegoprazan as well as vonoprazan, we suggest that our experiments reconfirmed the evidence that gastric acid inhibition mediates phase III contraction. However, the mechanism of the stimulation of phase III contraction by the gastric acid inhibition is remains to be elucidated. In the clinical setting, it is reported that an impairment of gastric phase III contraction is attenuated in functional dyspepsia (FD) patients and the dyspeptic symptoms in the postprandial state is improved once the gastric MMC was recovered (Takahashi, 2013). The property of tegoprazan to restore the phase III MMC contraction may be a new option for the therapeutics to improve the symptoms in FD patients.

P-CAB is the new class of drug for acid-related gastrointestinal diseases and positioned as a next generation of PPIs. A clinical evaluation of vonoprazan demonstrated its potent and long-acting efficacy in patients with GERD and peptic ulcer compared to those of currently available PPIs. *In vitro* and *in vivo* animal pharmacology studies of tegoprazan in this study demonstrated that tegoprazan has similar pharmacological properties to those of vonoprazan. Tegoprazan is the novel P-CAB that may provide a new option for the therapy of gastric acid-related diseases as well as of gastrointestinal motility-impaired diseases in clinical use.

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Authorship Contributions

Participated in research design: Takahashi, and Take

Conducted experiments and data analysis: Takahashi, and Take

Wrote or contributed to the writing of the manuscript: Takahashi, and Take

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Footnotes

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Figure Legends

Fig. 1 Structure of tegoprazan

Fig. 2 Inhibition of porcine, canine, and human H^+/K^+-ATPase and canine Na^+/K^+-ATPase by tegoprazan were tested with ion-leaky vehicle. Each enzyme preparations were incubated with tegoprazan at 37 °C for 30 min (50 min for human) and inorganic phosphate concentration in the reaction were analyzed with colorimetric method. Each value represents mean ± S.E.M. of three independent experiments.

Fig. 3 Mechanism of inhibition of porcine H^+/K^+-ATPase by tegoprazan was determined. (**A**) The enzyme kinetic analysis of the inhibition of porcine H^+/K^+-ATPase by tegoprazan was carried out with tegoprazan concentrations at 0, 0.15, 0.30, 0.45, and 0.60 μM, and KCl at 15, 21, 30, and 60 mM. The results were analyzed with Lineweaver-Burk plot using GraphPad Prism 5 for Windows. The graph presents an mean of three independent experiments. (**B**) Binding reversibility of tegoprazan to porcine H^+/K^+-ATPase was tested. H^+/K^+-ATPase was incubated with 3 μM or 1 μM of tegoprazan and then the tegoprazan concentration in reaction mixture was diluted to 0.15 or 0.05 μM without any other changes of the composition of reaction mixture.

The graph shows the inhibition of H^+/K^+-ATPase activity before and after the tegoprazan concentration. Each value represents mean ± S.E.M. of three independent experiments.

Fig. 4 Plasma and gastric distribution of tegoprazan were tested. (**A**) Plasma concentrations of tegoprazan in male dogs following single oral administration at 0.3, 3, and 30 mg/kg (n=2) were quantified using LC-MS/MS. The blood samples were collected at 0.25, 0.5, 1, 2, 4, 8, and 24 hrs post dosing. (**B**) Concentrations of tegoprazan in plasma and gastric juice at 5 or 16 hrs after oral administration at 1 or 3 mg/kg to HP dogs, respectively, were determined. Each value represents mean ± S.E.M. from 3 dogs.

Fig. 5 Inhibitions of gastric acid secretion by tegoprazan and omeprazole were tested. (**A**) Intravenous histamine infusion (80 μg/kg/hr) to the HP dog was dosed from 1 or 1.5 hrs before to 5 hrs after the tegoprazan treatment. Single dosing of tegoprazan 0.1, 0.3, and 1.0 mg/kg or vehicle was orally administrated and the gastric acid levels were determined. Each value represents mean + S.E.M. from 4 dogs. (**B**) Tegoprazan 3 mg/kg or vehicle was administrated orally and the histamine infusion was initiated at

14.5 hrs after the tegoprazan dosing. The gastric acid levels were monitored starting from histamine dosing to 21 hrs. Each value represents mean ± S.E.M. from 3 dogs. (**C**) Effect of tegoprazan on the gastric acid secretion at initial day of the treatment was compared to that after 5 days consecutive treatment. Tegoprazan (0.3 mg/kg, PO, QD) was administrated orally once daily for 5 days. Each value represents mean +/- S.E.M. from 3 dogs. (**D**) Omeprazole 0.6 mg/kg was dosed for 5 days in the same dog model and the gastric acid secretion were determined on Day 1 and 5. Each values represent mean ± S.E.M. from the results of 3 dogs.

Fig. 6 Effects of tegoprazan and vonoprazan on the intra-gastric pH were tested. (**A**) The gastric fistula dogs were treated with pentagastrin 6 μg/kg/hr subcutaneous infusion which results in the immediate decrease of gastric pH to ca. 2. Oral tegoprazan at 0.3, 1, and 3 mg/kg were dosed 1.5 hrs after the starting of pentagastrin administration and continued the pH analysis for 5 hrs with 5 min interval. (**B**) Effects of the oral single clinical corresponding dose of vonoprazan (0.4 mg/kg) and higher (1 mg/kg) on the gastric pH were tested. Each result represents mean of of pH from the results of 4 dogs.

Fig. 7 Effect of tegoprazan on the dog gastric motility was studied. (**A**) The dogs indwelling the force transducer was fasted overnight and determined the gastric antral motility. During a period of phase III MMC contraction is observed, a vehicle treatment right after the contraction signal did not show any change in phase III contraction pattern. Treatment of pentagastrin (PG) quickly terminated the phase III signals and initiated continuous and random frequency signals. Administration of oral tegoprazan at 3 mg/kg after the pentagastrin infusion restored the phase III contraction signals that were disappeared by the pentagastrin. Figures show time courses in three dogs. (B) The experiment was conducted with 1 mg/kg vonoprazan in the same dog model.

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Tables

TABLE 1

IC_{50} values of tegoprazan in *in vitro* studies

	H^+/K^+-ATPase				Na^+/K^+-ATPase
	Ion-leakey			Ion-tight	
	Porcine	Canine	Human	Porcine	Canine
Tegoprazan μM	0.47 (0.42, 0.52)	0.29 (0.27, 0.31)	0.52 (0.50, 0.53)	0.13 (0.12, 0.14)	>100

Data for H^+/K^+-ATPase shows mean (95% confidence interval) from three independent experiments. Three experiments for canine Na^+/K^+-ATPase resulted in IC_{50} >100 μM.

TABLE 2

% Inhibition of binding/function of various molecules by tegoprazan

Target Molecule		10 μM tegoprazan	Assay Reference	
		% inhibition	Compound	IC_{50} (nM)
Binding inhibition				
Adenosine	A_1 (h)	25	DPCPX	4.0
	A_{2A} (h)	8	NECA	19
	A_3 (h)	36	IB-MECA	2.1
Adrenergic	α_1 (r, non-selective)	3	Prazosin	0.45
	α_{2A} (h)	8	Yohimbine	6.6
	α_{2B} (h)	11	Yohimbine	9.8
	α_{2C} (h)	10	Yohimbine	0.94
	β_1 (h)	0	Atenolol	170
	β_2 (h)	6	ICI 118551	2.6
	β_3 (h)	-6	Cyanopindolol	140
Angiotensin	AT_1 (h)	-1	Saralasin	0.64
Benzodiazepine	BZD (r, central)	12	Diazepam	10
Cannabinoid	CB_1 (h)	3	CP 55940	1.3
	CB_2 (h)	23	WIN 55212-2	2.1
Channels	Ca^{2+} (r, L, DHP site)	19	Nirendipine	1.7
	Ca^{2+} (r, L, diltiazem site)	-5	Diltiazem	26
	Ca^{2+} (r, L, verapamil site)	-5	D 600	50
	Ca^{2+} (r, N)	21	ω-conotoxin GVIA	0.0015
	Na^+ (r, site 2)	31	Veratridine	4500
	SK^+_{Ca} (r)	-9	Apamiin	0.009
	Cl^- (r)	-2	Picrotoxinin	270
Cytokine	CXCR4 (h)	3	SDF-1α	0.04
	TNF-α (h)	-15	TNF-α	0.06
Cholecystokinin	CCK_A (h)	4	CCK-8	0.27
	CCK_B (h)	0	CCK-8	0.27
Dopamine	D_1 (h)	6	SCH 23390	0.75
	D_{2S} (h)	2	(+)butaclamol	5.2
	D_{2S} (h, agonist site)	-11	7-OH-DPAT	2.7
	D_3 (h)	2	(+)butaclamol	6.4

	$D_{4.4}$ (h)	3	Clozapine	56
Endothelin	ET_A (h)	-6	endothelin-1	0.099
	ET_B (h)	9	endothelin-3	0.061
GABA	$GABA_A$ (r)	-10	Muscimol	8.4
	$GABA_B$ (h)	-7	CGP 54626	12
Glutamate	AMPA (r)	1	L-glutamate	1300
	Kainate (r)	3	kainic acid	40
	NMDA (r)	1	CGS 19755	390
	Glycine (r, strychnine-insensitive)	-4	Glycine	290
Hormone	GR (h)	18	Dexamethasone	5.3
	TH (r)	-6	T_3	0.20
	Ghrelin (h, GHS)	8	Ghrelin	0.25
	Motilin (h)	6	Motilin	1.1
	Androgen (h)	-9	Methyltrienolone	2.5
	MCH_1 (h)	7	MCH	0.19
Histamine	H_1 (h)	-8	Pyrilamine	4.8
	H_2 (h)	20	Cimetidine	270
	H_3 (h)	4	Rα -Me-histamine	1.6
	H_4 (h)	-5	Imetit	4.2
Imidazoline	I1 (r)	-3	Rilmenidine	130
Leukotriene	LTB_4 (h, BLT_1)	-1	LTB_4	0.23
	LTD_4, (h, $CysLT_1$)	-2	LTD_4	1.3
Melanocoltin	MC_1 (r)	21	NDP-α-MSH	0.24
	MC_3 (h)	1	NDP-α-MSH	0.45
	MC_4 (h)	3	NDP-α-MSH	0.53
Melatonin	ML_1 (r)	13	Melatonin	0.16
	ML_2 (r)	81	Melatonin	40
MAO	MAO-A (r)	-3	Clorgyline	2.0
Muscarinic	M_1 (h)	0	Pirenzepine	6.3
	M_2 (h)	9	Methoctramiine	25
	M_3 (h)	-13	4-DAMP	0.28
	M_4 (h)	4	4-DAMP	0.63
	M_5 (h)	2	4-DAMP	0.37
Neurokinin	NK_1 (h)	-10	$[Sar^9,Met\ (O_2)^{11}]$-SP	0.36
	NK_2 (h)	-3	$[Nle^{10}]$-NKA(4-10)	13

Neuropeptide Y	Y_1 (h)	5	NPY	0.23
Nicotine	N (r, neuronal, α4β2)	1	Nicotine	8.5
	N (h, muscle-type)	5	α -bungarotoxin	4.9
Opioid	δ (r, non-selective)	5	Haloperidol	74
	δ2 (h, DOP)	15	DPDPE	1.9
	κ (r, KOP)	23	U 50488	0.61
	μ (h, MOP, agonist site)	-5	DAMGO	0.78
Phencyclidine	PCP (r)	2	MK801	3.4
PDE	Rolipram (r)	17	Rolipram	1.6
Serotonin	5-HT_{1A} (h)	7	8-OH-DPTA	0.63
	5-HT_{1B} (r)	10	Serotonin	13
	5-HT_{1D} (h)	6	Serotonin	2.4
	5-HT_{2A} (h)	33	Ketanserin	2.2
	5-HT_{2A} (h, agonist site)	1	(±)DOI	1.3
	5-HT_{2B} (h, agonist site)	46	(±)DOI	2.9
	5-HT_{2C} (h)	-1	RS-102221	5.5
	5-HT_{2C} (h, agonist site)	-2	(±)DOI	2.2
	5-HT_3 (h)	7	MDL 72222	8.2
	5-HT_{4e} (h)	36	Serotonin	480
	5-HT_6 (h)	14	Serotonin	230
	5-HT_7 (h)	-6	Serotonin	0.56
Somatostatin	sst_4 (h)	16	Somatostatin	7.3
Transporters	NE (h)	3	Protriptyline	12
	DA (h)	4	BTCP	11
	GABA (h)	-30	nipecotic aid	3200
	Choline (h, CHT1)	20	hemicholinium-3	6.3
	5-HT (h)	-4	Imipramine	4.0
Urotensin	UT1 (h)	-4	urotensin-II	0.95
Vasopressin	V_{1a} (h)	0	$[d(CH_2)_5^1,Tyr(Me)_2]$-AVP	0.97
	V_2 (h)	0	AVP	1.3
VIP	VIP_1 (h, $VPAC_1$)	0	VIP	0.25
Functional Inhibition				
Enzymes	COX-1 (h)	-19	Diclofenac	20
	COX-2 (h)	11	NS398	72
	PDE2 (h)	26	EHNA	4000

	PDE3 (h)	-13	Milrinone	160
	PDE4 (h)	35	Rolipram	580
	PDE5 (h)	9	Dipyridamole	1800
	PDE6 (h)	38	Zaprinast	190
	PDE11 (h)	14	Dipyridamole	490
	ACE (h)	-29	Captopril	3.6
	Caspase-3 (h)	-1	Ac-DEVD-CHO	0.48
	MMP-9 (h)	1	GM6001	0.61
	Carbonic anhydrase II (h)	-12	Acetazolamide	7.8
	Acetylcholinesterase (h)	3	Neostigmine	41
	ATPase (d, Na^+/K^+)*	4	Ouabain	490
kinase	FLT-1 (h, VEGFRK1)	8	Staurosporine	7.9
	p38α (h)	18	SB202190	31
	Abl (h)	3	Staurosporine	120
	CaMK2α (h)	4	AIP	430
	Lyn (h)	18	Staurosporine	48
	ZAP70 (h)	-9	Staurosporine	34

h, human origin; r, rat origin; d, dog; *, tegoprazan 30 μM

Data shows mean of two datapoints

TABLE 3

Pharmacokinetic parameters of tegoprazan in dog

Dose (mg/kg)	C_{max} (ng/mL)	T_{max} (hr)	$t_{1/2}$ (hr)	$AUC_{1\text{-}inf}$ (ng·hr/mL)
0.3	224 (11.0)	0.750 (0.433)	3.43 (0.132)	1181 (530)
3	2490 (420)	0.500 (0.00)	3.30 (0.759)	12731 (6180)
30	23833 (2438)	1.08 (0.878)	3.52 (0.280)	163951 (28969)

Data represent mean (SD) of 3 animals

Figures

Fig. 1

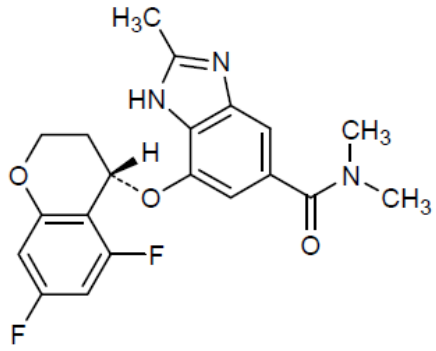

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Fig. 2

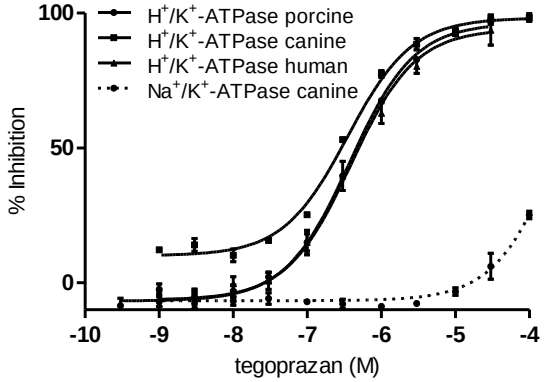

Fig. 3

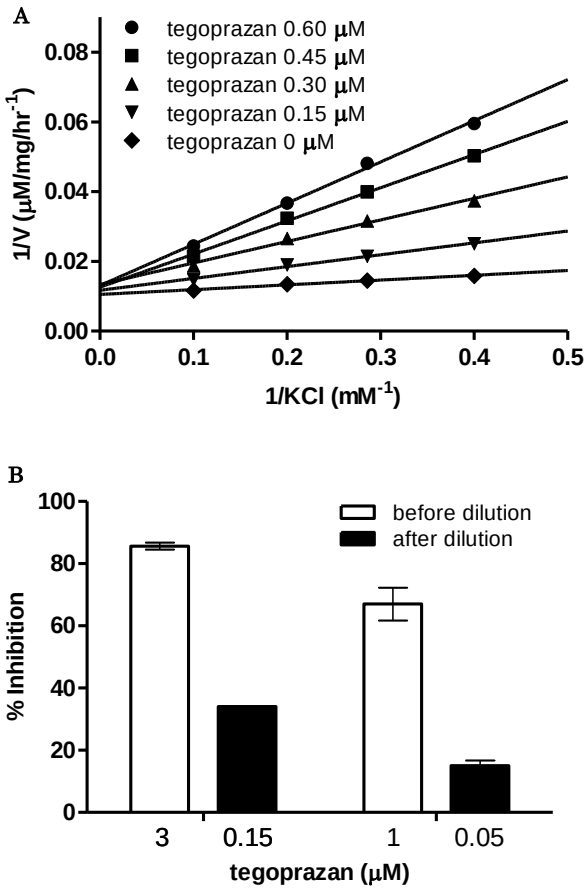

Fig. 4

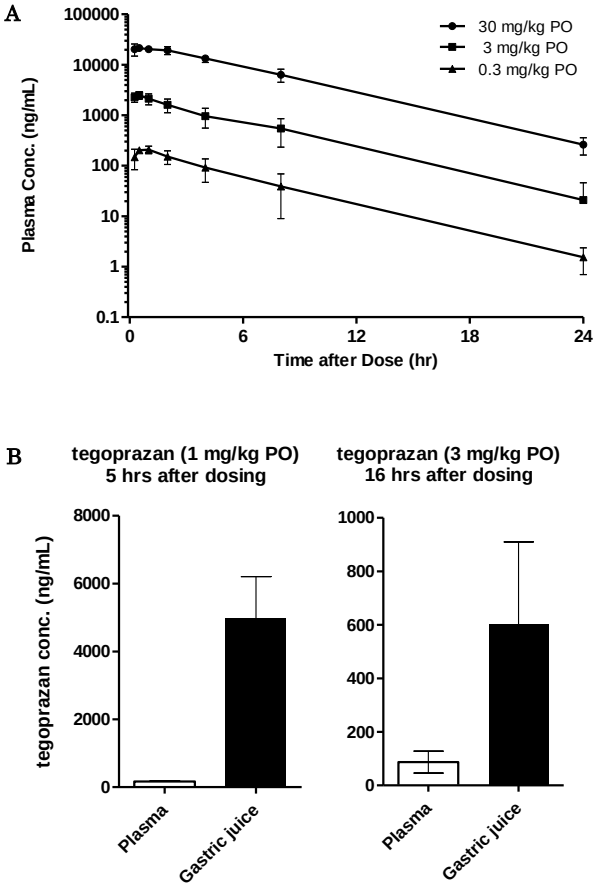

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Fig. 5

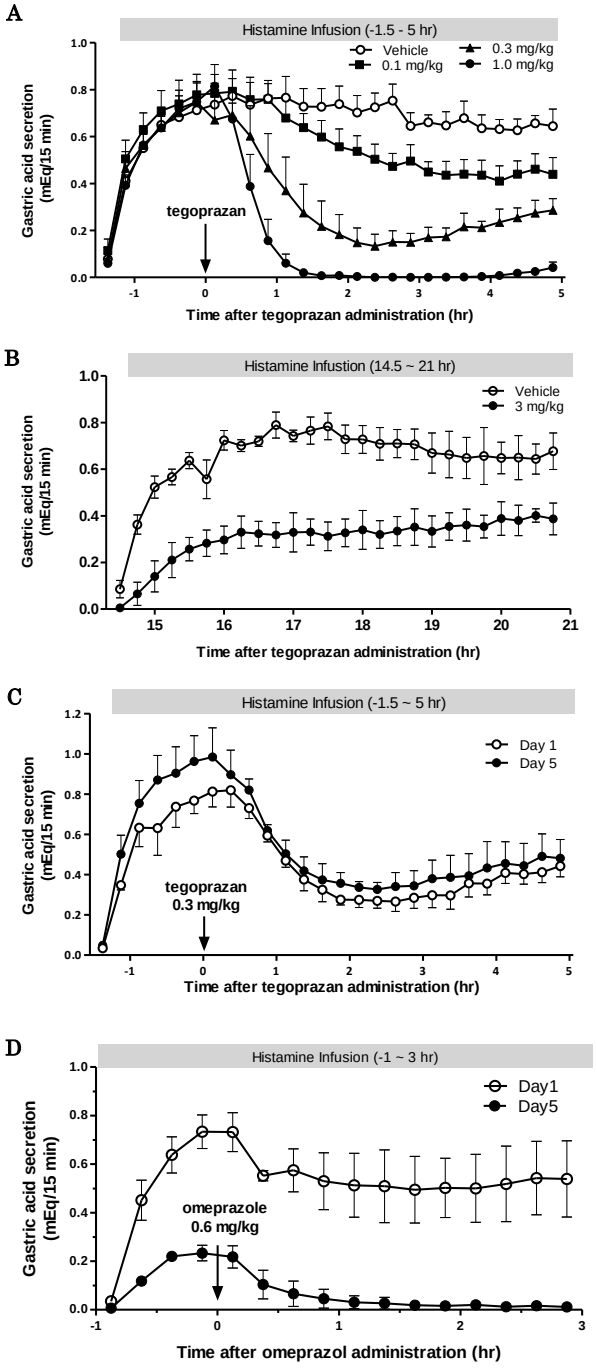

Fig. 6

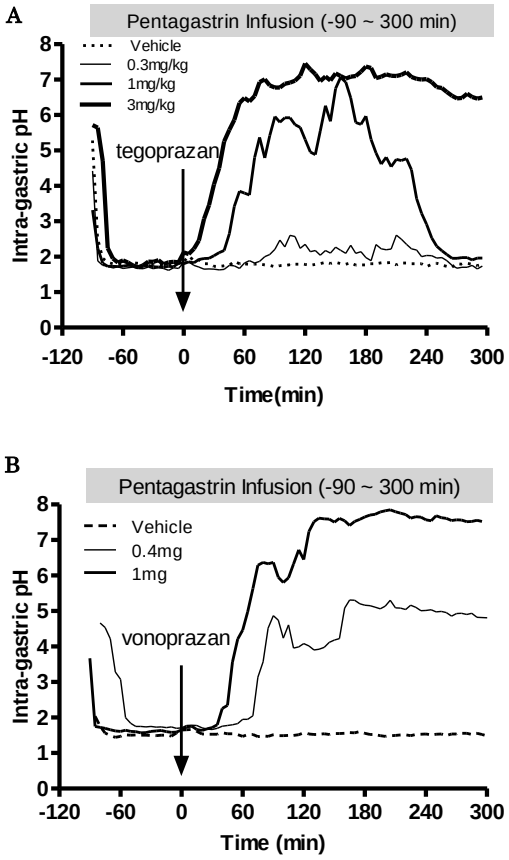

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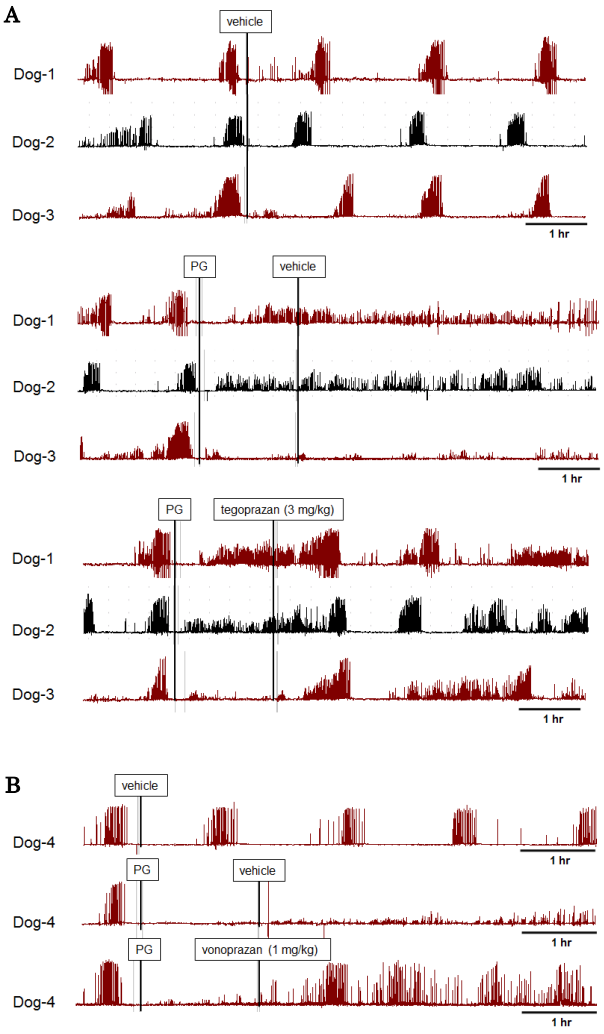
Fig. 7



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Supplemental Data

Not available