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Effects of tegoprazan, a novel potassium-competitive acid blocker,

on rat models of gastric acid-related disease

Dong Kyu Kim, Keun-Ho Lee, Sung-jun Kim, Soo-Jin Kim, Song Jin Lee, Chi Hye Park,

Bong-Tae Kim, Geun-Seog Song, Byoung-Seok Moon, Shin-Young Ryu

CJ HealthCare Corporation, Seoul, Korea

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ADDRESS CORRESPONDENCE TO:

Shin-Young Ryu, CJ HealthCare Co., Deokpyeong-ro 811, Majang-myeon, Icheon-si,

Gyeonggi-do 467-812, Republic of Korea. Phone: +82-31-639-4799; Fax: +82-31-632-2784;

E-mail: shinyoung.ryu@kolmar.co.kr

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ABBREVIATIONS:

AZD0865, 8-[(2,6-dimethylbenzyl)amino]-*N*-[2-hydroxyethyl]-2,3-dimethylimidazo[1,2-*a*]pyridine-6-carboxyamide; GERD, gastroesophageal reflux disease; H_2RA, Histamine receptor 2 antagonist; MPO, myeloperoxidase; NSAID, non-steroidal anti-inflammatory drug; P-CAB, potassium-competitive acid blocker; PPI, proton pump inhibitor; PUD, peptic ulcer disease; Revaprazan, 5,6-dimethyl-2-(4-fluorophenylamino)-4-(1-methyl-1,2,3,4-tetrahydroisoquinolin-2-yl)pyrimidine; SCH28080, 3-(cyanomethyl)-2-methyl,8-(phenylmethoxy)imidazo(1,2-a)pyridine; Tegoprazan, 7-[[(4S)-5,7-difluoro-3,4-dihydro-2H-chromen-4-yl]oxy]-N,N,2-trimethyl-3H-benzimidazole-5-carboxamide; Vonoprazan, 1-[5-(2-fluorophenyl)-1-(pyridine-3-ylsulfonyl)-1*H*-pyrrol-3-yl]-*N*-methylmethanamine monofumarate; WIRS, water immersion-restraint stress

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ABSTRACT

Tegoprazan, a novel potassium-competitive acid blocker (P-CAB), is a next-generation therapeutics developed for the treatment of acid-related gastrointestinal diseases such as gastroesophageal reflux disease (GERD) and peptic ulcers. In the present study, the *in vitro* and *in vivo* pharmacological properties of tegoprazan were compared with those of esomeprazole, a representative proton pump inhibitor. *In vitro* enzyme assays were performed using ion-leaky vesicles containing gastric H^+/K^+-ATPases isolated from porcine. The *in vivo* efficacies of tegoprazan were evaluated in rat models of GERD and peptic ulcer. Tegoprazan inhibited the activity of porcine H^+/K^+-ATPase with an IC_{50} value of 0.53 μM in a reversible manner, while esomeprazole showed weak and irreversible inhibition with an IC_{50} value of 42.52 μM. In a GERD model, tegoprazan showed dose-dependent efficacy in inhibiting esophageal injury and gastric acid secretion with an ED_{50} of 2.0 mg/kg, which was 15-fold more potent than that of esomeprazole. In peptic ulcer models, tegoprazan exhibited superior anti-ulcer activity compared to esomeprazole. The ED_{50} of tegoprazan in the naproxen-, ethanol-, and water-immersion restraint stress-induced peptic ulcer models were 0.1, 1.4, and 0.1 mg/kg, respectively. In the acetic acid-induced peptic ulcer model, the curative ratio of tegoprazan at 10 mg/kg was higher than that of esomeprazole at 30 mg/kg (44.2% versus 32.7%, respectively), following 5 days repeated oral administration. Thus, tegoprazan is a novel P-CAB that shows potent and reversible inhibition of gastric H^+/K^+-ATPase and may provide stronger efficacy compared to previous proton pump inhibitors.

Introduction

Acid-related diseases such as gastroesophageal reflux disease (GERD) and peptic ulcer disease (PUD) are common disorders in the upper digestive system associated with gastric acid secretion (Sachs et al., 2010). Drugs based on different mechanisms have been developed to control gastric acid secretion for the treatment of acid-related diseases or alleviate the related symptoms (Burget et al., 1990). Treatments for acid-related diseases have been revolutionized by drugs that exhibit improved efficacy and pharmacokinetic properties based on a new mechanism of acid suppression (Inatomi et al., 2016). Histamine-2-receptor antagonists (H_2RA) such as ranitidine are first-generation acid suppressants that have been widely used for more than 40 years (Feldman et al., 1990). However, the healing rates of H_2RA in GERD and PUD are only 50% and 74%, respectively, mainly because of insufficient suppression of gastric acid secretion (Robinson M., 2004). Proton pump inhibitors (PPIs), such as esomeprazole, are second-generation acid suppressants targeting gastric H^+/K^+-ATPase, the enzyme responsible for the final step of gastric acid secretion on the luminal side of gastric parietal cells (Modlin et al., 2004; Rabon et al., 1990). Because of its enhanced acid suppression effects, esomeprazole significantly increased the healing rate in GERD or PUD compared to H_2RA (Edwards et al., 2006). Thus, PPIs became a standard therapy for various acid-related gastrointestinal disorders (Frazzoni et al., 2003; Robinson, 2005). However, PPI therapy exhibits some limitations because of its mechanism of action and drug properties (Fass et al., 2005). PPIs as prodrugs require the acid activation process to convert into active

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sulfenamide forms, which are responsible for irreversible inhibition of H^+/K^+-ATPase via covalent binding (Sachs et al., 1988; Nagaya et al., 1989; Shin et al., 2002). Therefore, it is recommended that PPIs be taken before meals for the acid activation (Sachs et al., 1995). Moreover, because of the short plasma half-life of PPIs and the *de novo* cell surface insertion of H^+/K^+-ATPase, approximately 3–5 days are required to reach the maximum efficacy in gastric pH elevation (Katz et al., 2000; Ang et al., 2006). PPI efficacy is also affected by CYP2C19 polymorphism because most PPIs are substrates of CYP2C19 (Dammann et al., 1999; Furuta et al., 2005). Indeed, a field study suggests that 40% of patients with GERD responded poorly to standard PPI therapy, possibly because of insufficient suppression of gastric acid secretion (Cicala et al., 2013).

To overcome these limitations of PPI therapy, a new class of acid suppressants known as potassium-competitive acid blockers (P-CABs) has been developed (Andersson et al., 2005). Unlike PPIs, P-CABs directly inhibit gastric H^+/K^+-ATPase in a K^+-competitive and reversible manner, and thus do not require the acid activation process for inhibition (Vakil, 2004). Prototype P-CABs such as SCH28080 or AZD0865 are based on the imidazolopyridine structure and exhibit characteristics typical to P-CABs, including potent and reversible K^+ competitive inhibition of H^+/K^+-ATPase with rapid onset of action (Beil et al., 1986). However, the development of SCH28080 and AZD0865 was suspended because of hepatotoxicity concerns (Parsons and Keeling, 2005). Revaprazan, based on the pyrimidine-2 amine structure, is the first P-CAB marketed in South Korea. However, the acid suppression

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ability and gastric pH>4.0 holding time of revaprazan is not superior to conventional PPIs (Kim HK et al., 2010). Vonoprazan, a pyrrole derivative recently marketed in Japan, exhibits superior acid suppression compared to PPIs (Karly et al., 2015). However, safety concerns for the long-term use of vonoprazan have been raised because of significant elevation of serum gastrin levels compared to that of conventional PPI therapy, possibly because of its high pKa value (>9) and strong inhibition of acid secretion (Ashida et al., 2016; Miwa et al., 2017; Murakami et al., 2016). The elevated serum gastrin level is closely correlated with the increased incidence of gastric endocrine cell tumors in a nonclinical carcinogenicity study (Cappiello and Layton, 1965). However, the safety risk of elevated gastrin levels due to vonoprazan in humans remains unclear.

Tegoprazan (Fig. 1), a novel P-CAB based on the benzimidazole structure, is a potent and highly selective inhibitor of H^+/K^+-ATPase with characteristics typical to P-CAB. Tegoprazan exhibited potent inhibition of acid secretion in the Heidenhain pouch dog model with rapid onset of maximal efficacy (Takahashi and Take, 2017).

In the present study, we demonstrate that tegoprazan effectively controls gastric acid-related diseases using animal models of GERD and PUD. The *in vitro* and *in vivo* pharmacological characteristics were compared with those of esomeprazole, a representative PPI.

Materials and Methods

Chemicals

Tegoprazan (CJ HealthCare, Republic of Korea), esomeprazole (Shouguang, China) and SCH28080 (S4443, Sigma) were dissolved in dimethyl sulfoxide (DMSO) to assess the *in vitro* activity of H^+/K^+-ATPase. For *in vivo* experiments, tegoprazan and esomeprazole were dissolved in 0.5% methyl cellulose solution. Drugs were administered to animals via the oral route or intraduodenal injection. All the reagents and solvents used in this study were of analytical grade or higher.

Animals

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the CJ HealthCare animal ethics committee (No. CJ-0196-00, CJ-0234-00, CJ-0310-00, CJ-0311-00). Porcine stomachs were obtained from a local abattoir (Daesung Corporation, Republic of Korea). Male Sprague-Dawley (SD) rats were purchased from Han Lym Lab. (Republic of Korea).

Preparation of porcine gastric H^+/K^+-ATPase

Vesicles containing H^+/K^+-ATPases were isolated from fresh porcine stomachs as previously described (Takahashi and Take, 2017). Porcine stomachs were dissected alongside the greater curvature, followed by washing with ice-cold phosphate-buffered saline. The gastric mucosal

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layer was scraped off from the underlying muscular layer and then added to lysis buffer

containing 250 mM sucrose, and 40 mM HEPES, followed by homogenization on ice for 20

min. The homogenate was centrifuged at 20,000 $\times g$, 4℃ for 30 min, and the supernatant was

further centrifuged at 115,000 $\times g$, 4℃ for 30 min. The pellets were resuspended in lysis

buffer and centrifuged at 130,000 $\times g$, 4℃ for 1 h. The pellets were resuspended in dH_2O and

immediately frozen in liquid nitrogen. To produce lyophilized vesicles, the resuspended

vesicles were permeabilized by freeze-drying processing and then stored at −70℃. Prior to

use, gastric vesicles were thawed by adding an equal volume of dH_2O. The final

concentration of gastric vesicles was 3 μg/μL.

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

 The H^+/K^+-ATPase activities were determined by colorimetric measurement of inorganic

phosphate production in a reaction mixture containing isolated porcine gastric vesicles as

described previously (Mori et al, 2009; Keeling et al, 1998). The porcine gastric H^+/K^+-

ATPase activities were measured in a 60 μL reaction mixture containing 4.5 μg of porcine

ion-leaky vesicles in a reaction buffer (20 mM KCl, 3 mM MgSO4, 3 mM Na_2ATP, 40 mM

Bis-tris, pH 6.4) containing various concentrations of tegoprazan or esomeprazole at 37℃ in

a 96-well polystyrene plate. For 0% or 100% inhibition controls, the enzyme reaction was

performed in the presence of 1% DMSO (a vehicle control) or 100 μM SCH28080, a

selective inhibitor of gastric H^+/K^+-ATPase. The reaction was started by adding Na_2ATP and the mixture was incubated at 37℃ for 30 min, and then 30 µL of 10% sodium dodecyl sulfate containing antifoam A was added to stop the reaction. Inorganic phosphates were measured in 200 µL of colorimetric reagent consisting of 4 parts of 10% L-ascorbic acid, pH 5 and one part of 35 mM ammonium molybdate in 15 mM zinc acetate, pH 5. Following incubation with the colorimetric reagent at 37℃, the optical density was measured at 750 nm with a plate reader (Power wave XS, Bio-TEK, USA). The standard inorganic phosphate solution was prepared using KH_2PO_4. Tegoprazan and esomeprazole were dissolved in DMSO to concentration of 10 mM, and then diluted in the reaction mixture to the appropriate final concentrations.

Reversibility of H^+/K^+-ATPase activity

To examine the reversibility of the tegoprazan or esomeprazole-induced H^+/K^+-ATPase inhibition, the enzyme activities before and after washout of drugs were compared. Porcine gastric H^+/K^+-ATPase activities were measured in a 600 µL reaction mixture containing 60 µg of porcine ion-leaky vesicles and reaction buffer (5 mM KCl, 3 mM $MgSO_4$, 3 mM Na_2ATP, 40 mM Bis-tris, pH 6.4) at 37℃ in a 96-well polystyrene plate. The reaction was started by adding Na_2ATP and the mixture was incubated at 37℃ for 40 min. After incubation, 60 µL of the solution was collected, followed by the measurement of H^+/K^+-

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ATPase activity as described above. The remaining 540 μL was centrifuged at 27,800 ×*g*, 4℃

for 60 min. After removing the supernatant, the pellet was resuspended in distilled water prior

to further centrifugation at 27,800 ×*g*, 4℃ for 20 min. After removing the supernatant, 600

μL of the reaction mixture, not containing gastric vesicles, was added to the pellet. The final

mixture was incubated at 37℃ for 80 min. Following the incubation, 60 μL of the solution

was collected for each compound, and H^+/K^+-ATPase activity was measured.

Measurement of gastric acid secretion in pylorus-ligated rats

Drugs were orally administered to 5–6 rats after fasting for 24 h. Vehicle control group was

also used at 0.5% methylcellulose. After 30 min of drug administration, the pylorus was

ligated under weak anesthesia (3% isoflurane inhalation) and the abdomen was closed by

surgical suturing. After 5 h of pyloric ligation, the rats were sacrificed and the stomachs were

removed. The gastric contents were collected and centrifuged at 3,000 rpm for 5 min. The pH

of the gastric contents was measured using an automatic titrator (Orion 3 star, Thermo

scientific Co.). For multiple dosing experiment, drugs were orally administered daily for 4

days and the fifth dosing was administered after fasting for 24 h. The pylorus-ligated model was

prepared using a single administration design.

Induction of reflux esophagitis in rats

The rat reflux esophagitis model was induced as described previously (Rao CV and

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Vijayakumar M, 2008). Rats were fasted for 24 h, anesthetized by inhaling 3% isoflurane (HARVARD), and the stomach was exposed after a 1 cm incision was made at the left upper quadrant of the abdomen. The duodenal pyloric ring and the border between the forestomach and glandular area of the exposed stomach were ligated by surgical suture. After ligation of the pyloric ring, each drug was intraduodenally administered at 5 mL/kg using 3 mL syringes. The stomach was returned to its original position, and the abdomen was closed using autoclips. After surgery, rats were not allowed to eat or drink for 4 h. Rats were then sacrificed by CO_2 asphyxiation and their abdomens were incised. The esophagus was exposed up to the neck region, and the portions of esophagus near the neck and just below the ligated pylorus near the duodenum were cut to excise both the esophagus and stomach. The gastric contents were collected and centrifuged at 3,000 rpm for 5 min. Using the supernatant, the amount and pH of the gastric juice were measured, and total acid output was calculated. The stomach was incised along the greater curvature extending to the esophagus, and a photo was acquired with a digital camera. The esophagus epithelial tissues were scraped using a slide glass on ice. After adding lysis buffer (200 mM NaCl, 10 mM Tris, 5 EDTA, 1 mM PMSF, and 10% glycerin, 1% protease inhibitor cocktail), the tissue was lysed for 1 h on ice. The lysate was centrifuged for 1,500 $\times g$, 4℃ for 15 min, and the supernatant was collected for the protein assay. The amount of myeloperoxidase in 100 μg of protein was measured according to the MPO kit instructions (RAT MPO ELISA KIT, Hycult biotech).

Induction of peptic ulcer in rats

NSAID-induced peptic ulcer model. Rats were fasted for 24 h before oral administration of tegoprazan and esomeprazole. One hour after drug administration, naproxen (30 mg/kg) was orally administered to each rat for 3 times at 2-h intervals. The rats were sacrificed at 4 h after the final naproxen administration. The stomach was collected and fixed in 2% formalin on ice for 1 h. The great curvature of each stomach was incised and photographed using a digital camera. The ulcer area was analyzed with an image analyzer (Image J, National Institutes of Health) and the area of the lesion was summed for each rat and the ulcer index (%) was calculated per total area of the stomach. For multiple dosing experiment, tegoprazan and esomeprazole were orally administered daily for 4 days and the fifth dosing was administered after fasting for 24 h. The naproxen-induced peptic ulcer model was prepared in a single administration design.

Ethanol-induced peptic ulcer model. Rats were fasted for 48 h before oral administration of tegoprazan and esomeprazole. One hour after drug administration, absolute ethanol (5 mL/kg) was orally administered to each rat. The rats were then sacrificed at 1 h after ethanol administration, and the area of gastric ulcers was calculated using as a described above.

Water-immersion restraint stress (WIRS)-induced peptic ulcer model. WIRS is commonly used as an experimental model of stress-mediated acute gastric ulcer lesions because of its clinical relevance and reproducibility. Rats were fasted for 48 h before oral administration of tegoprazan and esomeprazole. Ten minutes after drug administration, the

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rats were placed in a restraint cage and immersed in water at 21℃ for 4 h. The rats were

sacrificed 4 h later, and the area of gastric ulcers was calculated as described above.

Acetic acid-induced peptic ulcer model. Gastric ulcers (namely, kissing ulcers) were

induced by intraluminal exposure to acetic acid. Before surgery, the rats were fasted

overnight with free access to water. On the next day, rats were anesthetized with

xylazine/ketamine (7.5 mg/kg and 60 mg/kg) by intraperitoneal administration and the

stomach was exposed via midline epigastric incision. The antrum was clamped using a

sponge forceps (inner diameter, 6mm) and 100 μL of 60% acetic acid was then injected into

the clamped area for 40 s. The incision was then closed by suturing. Each animal was allowed

to recover from anesthesia. Two days after the surgical procedure, tegoprazan and

esomeprazole were orally administered daily for 5 days. Animals were fasted for 24 h before

the sacrifice. After anesthesia, the stomach was collected from each animal, and then opened

along the greater curvature, rinsed with saline, pinned out on a cork board, and photographed.

The total ulcerated area (mm^2) was measured using Leica Application Suite V4 software

(Leica Microsystems, Germany). The curative ratio was calculated as follows: curative ratio

(%) = {(Ulcer area of control group − ulcer area of test group) / ulcer area of control group}

× 100

Stomach distribution study in rats

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Dosing and sample collection. Tegoprazan was orally administered at a dose of 10 mg/kg to fasted Sprague-Dawley male rats. The blood and stomach were collected at 0.25, 0.5, 1, 6, 12, and 24 h after dosing. Blood was immediately centrifuged to obtain the plasma, and stomach tissue was weighed and rinsed with physiological saline to remove the blood or contents. All plasma and tissue samples were store at -20°C until analysis.

Measurement of Tegoprazan. A 100 μL of plasma sample added with 20 μL of internal standard (donepezil, 1 μg/mL in 50% methanol) and 1 mL of acetonitrile to induce protein precipitation. The mixture was vigorously mixed and centrifuged, and then 100 μL of the supernatant was added with 700 μL of 0.1% formic acid in water-acetonitrile (50:50, v/v) and mixed by vortexing. After centrifugation, 5 μL of the supernatants of each sample was directly injected into a liquid chromatography-tandem mass spectrometry system. Each stomach sample was thawed and mixed with ice-cold distilled water at 1:1 (g/mL). After processing in a homogenizer, 100 μL of each tissue homogenate was added to 20 μL of internal standard and 1 mL of acetonitrile. Next, the tissue samples were treated in the same manner as the plasma sample.

Chromatography was performed on a reversed-phase column (Cadenza CW-C18 column; 2.0 ×75 mm, 3 μm; Imtakt, Portland, OR) using isocratic elution with a mobile phase of 0.1% formic acid in water-acetonitrile (70:30, v/v) at a flow rate of 0.2 mL/min. The column and autosampler tray temperature were maintained at 35°C and 4°C, respectively. Mass spectrometry was operated in positive trubo-ion spray model (API5000, AB Sciex, CA, USA)

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at the transition (m/z) 338.3 to 220.3 for tegoprazan and 380.4 to 91.2 for internal standard.

The limit of quantification was 10 ng/mL for tegoprazan with acceptable precision (coefficient of variation <15%) and accuracy (relative error <15%).

Pharmacokinetics. Non-compartmental pharmacokinetic analysis of all data was performed using WinNonlin 5.2 (Pharsight, Cary, NC).

Data analysis

Data are presented as the mean ± S.D. or S.E. IC_{50} or ED_{50} values were obtained from the logistic fit of dose response data (PRISM 5, Graphpad software, USA). In the reversibility test and GERD model, the differences between each drug treatment group and control group were tested for statistical significance by Student's *t*-tests. *p* values less than 0.05 were considered statistically significant. In the gastric acid secretion experiment and peptic ulcer models, the differences between each drug treatment group and vehicle group were tested for statistical significance by the non-parametric Mann-Whitney *U* tests. *p* values less than 0.05 were considered statistically significant.

<div align="center">

Results

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Effects of tegoprazan and esomeprazole on porcine gastric H^+/K^+-ATPase activity.

Because direct and reversible inhibition of H^+/K^+-ATPase are critical characteristics of P-

CABs, the inhibition potency and recovery of H^+/K^+-ATPase activity following washout of the test drugs were evaluated using porcine H^+/K^+-ATPase containing vesicles under ion-leaky condition. Because the potassium binding site of the H^+/K^+-ATPase is inside the vesicle in this preparation, potassium ion outside the vesicle cannot access the ATPase in the absence of an ionophore. In the ion-leaky vesicle, potassium can freely access the potassium-binding site in the assay solution (Takahashi and Take, 2017). Under the ion-leaky condition, both compounds inhibited H^+/K^+-ATPase activity in a dose-dependent manner. The IC_{50} values of tegoprazan and esomeprazole were 0.53 and 42.52 μM, respectively. The inhibitory activity of tegoprazan was 80-fold higher than that of esomeprazole (Fig. 2A). We also tested the reversibility of the inhibitory potency of both compounds on the H^+/K^+-ATPase activity. The assay of dose-dependent inhibition by tegoprazan and esomeprazole on the H^+/K^+-ATPase activity showed that the concentration causing approximately 50% of equal inhibitory effects were 1 and 40 μM, respectively. When the H^+/K^+-ATPase activity of the control group was set as 100% before the washout, the activity of the control group after the washout was 95.9%. Before washout, tegoprazan (1 μM) showed a percent control activity of 61.5% compared to the control. After washout, the H^+/K^+-ATPase activity level returned to that of the control, demonstrating the reversibility of the inhibitory mechanism. In contrast, esomeprazole (40 μM) showed a percent control activity 56.9% compared to the control before the washout and exerted 32.0% activity even after washout (Fig. 2B). These results are in agreement with previous studies showing that the inhibitory effects of PPIs are irreversible.

Effects of tegoprazan and esomeprazole on gastric acid secretion in pylorus-ligated rats.

This study was conducted to test the inhibitory potency of tegoprazan and esomeprazole on

gastric acid secretion through pylorus ligation in conscious rats. Because esomeprazole may

require several days of dosing to reach full efficacy, the drugs were evaluated against each other in

single and five-day repeated dosing designs. The basal gastric pH values of the vehicle control

for 5 h after the surgical procedure were pH 1.39 and 1.51 at single dosing and 5 consecutive

days, respectively. For the single and multi-day dosing regimen, the gastric pH values of 3 mg/kg

tegoprazan were 4.13 and 4.34 and for 10 mg/kg tegoprazan were 6.86 and 6.63, respectively. Thus,

tegoprazan inhibited basal gastric acid secretion in a dose-dependent manner and was effective at a

single dose (Fig. 3A). For the single and multi-day dosing regimen, the gastric pH values of 10

mg/kg esomeprazole were 1.95 and 2.98 and for 30 mg/kg esomeprazole were 4.86 and 6.02,

respectively. Esomeprazole also inhibited basal gastric acid secretion in a dose-dependent manner

and then showed full efficacy in multi-day dosing regimen (Fig. 3B). Tegoprazan completely

inhibited gastric acid secretion at the 10 mg/kg single dosing, whereas inhibition by

esomeprazole was incomplete, even for the 30 mg/kg multi-day dosing regimen.

Effects of tegoprazan and esomeprazole on rats with experimental reflux esophagitis. By

dual ligation at the duodenal pyloric ring and the forestomach, we prepared a rat model to

induce the reflux of gastric contents into the esophagus. We observed esophageal mucous

membrane damage from the low pH of the refluxed gastric contents. In this study, the

inhibitory effects of tegoprazan on esophageal mucosa injury and gastric acid secretion were

evaluated and compared to those of esomeprazole. Severe longitudinal hemorrhagic lesions with blackish exudates were observed in the esophagus of the reflux esophagitis vehicle group. However, tegoprazan at doses of 0.3 and 1 mg/kg showed reduced esophagitis area with mild hemorrhagic lesions; tegoprazan at doses of 3 to 30 mg/kg showed no lesions in reflux esophagitis. Esomeprazole at doses of 0.3 and 1 mg/kg were not effective for decreasing reflux esophagitis, and higher doses of 3–30 mg/kg showed no lesions in reflux esophagitis (Fig. 4A). Gastric total acid output in the vehicle group was 114 mEq/L per 5 h, and the 0.3–30 mg/kg tegoprazan inhibited gastric acid output in a dose-dependent manner (Fig. 4B) with an ED_{50} of 2.0 mg/kg (Table 1). Esomeprazole exerted an inhibitory effect only at the high dose of 30 mg/kg (Fig. 4B), with an ED_{50} value of 32.5 mg/kg (Table 1). Damage to the esophageal mucous tissue caused by gastric acid reflux was measured from the amount of MPO in the esophageal mucous tissue. The vehicle group contained 820 ng of MPO per 100 μg of protein, whereas both the tegoprazan and esomeprazole groups showed reduced MPO levels in a dose-dependent manner (Fig. 4C), with ED_{50} values of 5.9 and 29.2 mg/kg, respectively (Table 1).

Effects of tegoprazan and esomeprazole on rats with naproxen-induced gastric ulcer.

This study was conducted to evaluate the anti-ulcer efficacy of tegoprazan and esomeprazole in a naproxen-induced acute gastric ulcer rat model. Because esomeprazole may require several days of dosing to reach full efficacy, the drugs were evaluated against each other in single and five-day repeated dosing designs. The ulcer lesions (%) were calculated by dividing the area of the

ulcer lesion per total area of the stomach. For the single and multi-day dosing regimen, the ED_{50} value of tegoprazan at 0.1, 1, and 10 mg/kg were 0.1 and 0.1 mg/kg, respectively. Thus, tegoprazan exerted anti-ulcer effect in a dose-dependent manner, and was effective at a single dose. The ED_{50} values of esomeprazole for the single and multi-dosing regimen were 3.9 and 0.9 mg/kg, respectively (Table 2, Fig. 5A). This model also confirmed that the efficacy of esomeprazole was increased by repeated administration rather than single-dose administration. Despite the multi-dosing regimen, tegoprazan was 9-fold more potent than esomeprazole. In terms of the gross appearance of gastric ulcers, the vehicle group produced a punctuate type ulcer that was clean and deep. However, tegoprazan at a dose of 1 mg/kg, resulted in significantly reduced ulcer lesions with a shallower depth. Esomeprazole at a dose of 3 mg/kg also reduced ulcer lesions, but not significantly (Fig. 5A).

Effects of tegoprazan and esomeprazole on rats with ethanol-induced gastric ulcer. We assessed the effects of tegoprazan and esomeprazole on the acute damage to the gastric mucous due to administration of absolute ethanol. The calculation of ulcer lesions (%) followed the naproxen ulcer model. Tegoprazan at doses of 0.1, 1, and 10 mg/kg exerted a anti-ulcer effect in a dose-dependent manner with an ED_{50} of 1.4 mg/kg. Esomeprazole also suppressed gastric ulcer in a dose-dependent manner at 0.3, 3, and 30 mg/kg, with an ED_{50} of 7.3 mg/kg (Table 2, Fig. 5B). In terms of macroscopic appearance of gastric ulcers, the vehicle group produced elongated bands of severe hemorrhagic lesions parallel to the long axis of the stomach. Tegoprazan at a dose of 1 mg/kg significantly reduced the ulcer area and

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severity of hemorrhagic bands. However, esomeprazole at a dose of 3 mg/kg did not reduce

ulcer lesions (Fig. 5B).

Effects of tegoprazan and esomeprazole on rats with WIRS-induced gastric ulcer. We

assessed the effects of tegoprazan and esomeprazole against gastric ulcer induced by WIRS

for 4 h. The ulcer lesions (%) were calculated as described for the naproxen ulcer model.

Tegoprazan at doses of 0.1, 1, and 10 mg/kg exerted an anti-ulcer effect in a dose-dependent

manner with ED_{50} of 0.1 mg/kg. Esomeprazole suppressed gastric ulcer at only 30 mg/kg

with ED_{50} of 9.7 mg/kg (Table 2, Fig. 5C). For the macroscopic appearance of gastric ulcers,

the vehicle group showed serious gastric bleeding erosions, as indicated by mucosal

hemorrhage and mucosal erosive lesions. Hemorrhage was observed mainly in the gastric

body and antrum. However, tegoprazan at dose of 1 mg/kg showed a significantly reduced

hemorrhage area, while esomeprazole at dose of 3 mg/kg did not reduce hemorrhage lesions

(Fig. 5C).

Effects of tegoprazan and esomeprazole on rats with acetic acid-induced gastric ulcer.

To evaluate the effects of tegoprazan and esomeprazole on acetic acid-induced gastric ulcer

models, the ulcer lesions and curative ratio (%) were evaluated. Intraluminal application of

60% acetic acid produced 2 round deep gastric ulcer in the stomach of vehicle group, with the

ulcer area calculated as 14.46 mm^2. Oral administration of tegoprazan (0.1, 1 and 10

mg/kg/day for 5 consecutive days) significantly reduced the gastric ulcer area to 9.5, 8.9, and

8.1 mm^2, respectively. The curative ratio of tegoprazan was dose dependently increased by

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34.2%, 38.0%, and 44.2%. Esomeprazole (30 mg/kg) also reduced the gastric ulcer area to

9.7 mm^2 and the curative ratio of esomeprazole was 32.7% (Fig. 6A). The total ulcerated area

(mm^2) was measured using Leica application suite V4 software (Fig. 6B).

Stomach distribution of tegoprazan in rats. The concentration-time profiles of tegoprazan

in the plasma and stomach after oral administration of tegoprazan at a dose of 10 mg/kg are

shown in Fig. 7. Tegoprazan reached a Cmax at 0.25 h in both the blood and stomach, and the

concentration of tegoprazan in stomach was higher than that in the plasma at all sampling

times; additionally, tegoprazan was present in the gastric tissue 24 h after administration to

rats. This indicated that tegoprazan is very rapidly absorbed and distributed into the stomach.

Discussion

Recently, the basic pharmacological properties of tegoprazan, such as its potent and

selective inhibition of gastric H^+/K^+-ATPase and *in vivo* inhibitory efficacy for gastric acid

secretion in a Heidenhain pouch dog model, were reported (Takahashi and Take, 2017). In

this study, we further demonstrated that tegoprazan inhibits H^+/K^+-ATPase with 80-fold

higher potency than esomeprazole under ion-leaky conditions at pH 6.4. Under these

conditions, the direct inhibitory effect on H^+/K^+-ATPase can be compared without the

potential for drug accumulation in the acidic compartment or an acid activation process. In a

previous report, tegoprazan was 3-fold more potent in inhibiting H^+/K^+-ATPase activity under

ion-tight conditions than under ion-leaky conditions, suggesting that tegoprazan, a weakly

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basic compound with a pKa of 5.1, can be concentrated in acidic compartments (Takahashi

and Take, 2017). Moreover, the concentration of tegoprazan in stomach was higher than that

in the plasma, and 152 ng/g remained in the stomach at 12 h after oral administration at a

dose of 10 mg/kg (Fig. 7). The stomach is the target organ of tegoprazan, and its rapid

distribution into the stomach may be advantageous for achieving the pharmacodynamics

effect of tegoprazan. Therefore, these pharmacological properties of tegoprazan suggest that

it may be a more potent and effective agent for inhibiting gastric acid secretion *in vivo*

compared to esomeprazole. Another important characteristic of tegoprazan that differentiates

it from esomeprazole is the reversibility of its inhibition of H^+/K^+-ATPase activity. The

inhibitory activity of esomeprazole remained the same after washout, reflecting the

irreversible covalent nature of PPI binding to gastric H^+/K^+-ATPase. In contrast, the

inhibitory effect of tegoprazan was reversed by washout. This ensures that gastric acid

suppression was not unnecessarily prolonged when stopping the administration of tegoprazan,

thereby decreasing the risk of side effects such as acid rebounding. Previous Linweaver-Burk

plot analysis show that P-CABs such as tegoprazan and SCH28080 inhibit gastric H^+/K^+-

ATPase by competing with potassium ions (Takahashi and Take, 2017; Hori et al., 2010).

These findings confirm that tegoprazan is a P-CAB that does not form covalent bonds and

whose mechanism of H^+/K^+-ATPase inhibition, in contrast to that of PPIs, is reversible and

based on competition with potassium.

The *in vivo* anti-secretory effect of tegoprazan was investigated and compared with that of

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esomeprazole in pylorus-ligated rats. Tegoprazan at a single dose of 10 mg/kg completely inhibited basal acid secretion, whereas esomeprazole only achieved a pH of 6 even with the multi-day dosing regimen of 30 mg/kg. Notably, inhibition of acid secretion by tegoprazan was complete, likely because acid activation was not required for tegoprazan to exert its inhibitory effect, in contrast to esomeprazole, which requires acid activation. The inhibitory effect of tegoprazan on H^+/K^+-ATPase at pH 6.4 *in vitro* was approximately 80-fold greater than that of esomeprazole (Fig 2A), but the anti-secretory effect of tegoprazan *in vivo* was only approximately 5−10-fold more potent than that of esomeprazole (Fig 3). This discrepancy between the *in vitro* and *in vivo* finding can be explained by the difference in pH conditions. The pH under the *in vitro* conditions was 6.4 (almost neutral), whereas that of secretory canaliculi of the parietal cells *in vivo* was 1−2. Because esomeprazole is fully transformed into its active form under acidic conditions, its anti-secretory activity is more potent *in vivo* than *in vitro*. However, tegoprazan showed more potent inhibitory activity than esomeprazole.

For the treatment of GERD, attaining and maintaining an intragastric pH of >4 is important (Kahrilas et al., 2007), as the rate of healing of reflux esophagitis and onset of rapid symptom relief are associated with an intragastric pH of >4 (Bell et al., 1992). We prepared an experimental model of reflux esophagitis for only 4 h to compare the effects of rapid symptom relief after dual ligation at the duodenal pyloric ring and forestomach. The ED_{50} value of the total acid output of tegoprazan was 16-fold potent than that of esomeprazole.

This potent inhibition of acid secretion by tegoprazan can be explained by its ability to

maintain its activity unaffected by the ambient pH, combined with the potent activity of

tegoprazan itself. P-CABs are instantly protonated in an acidic environment and bind to and

inhibit H^+/K^+-ATPase in this protonated form (Andersson and Carlsson, 2005). The pKa

value of tegoprazan is 5.1, whereas that of esomeprazole is 4.0. Therefore, tegoprazan can

achieve a higher concentration than esomeprazole in acidic environments such as the

secretory canaliculi of the gastric parietal cells. Furthermore, PPIs are activated under acidic

conditions, and the active compound can inhibit only the H^+/K^+-ATPase that has already been

transferred to the apical membrane and activated (Forte and Yao, 1996). The resting proton

pumps are internalized in a tubulovesicle that is not transferred to the apical membrane.

Therefore, PPIs cannot inhibit these resting proton pumps because the active form of PPIs

cannot penetrate the membrane (Nagaya et al., 1990). Moreover, inhibition of acid secretion

by PPIs was decreased because of the short plasma $t_{1/2}$ and its rapid degradation in the

secretory canaliculi. However, P-CABs are unaffected by ambient pH and can inhibit H^+/K^+-

ATPase under both acidic and neutral conditions, indicating that P-CABs can bind to both

activated and resting acid pumps (Scott et al., 2015). Thus, tegoprazan may be able to achieve

maximal acid suppression from the first dosing, and its onset time is much more rapid than

those of PPIs such as esomeprazole. A previous study showed that vonoprazan, another P-

CAB, exerts a more potent and longer-lasting inhibitory effect on gastric acid secretion but is

unaffected by ambient pH in rats and dogs. However, because the pKa value of vonoprazan is

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9.3, it exists in many protonated forms under neutral pH conditions; thus, compared to other

P-CABs such as tegoprazan, it may take slightly longer for vonoprazan to reach its target site,

such as the gastric H^+/K^+-ATPase in parietal cell canaliculi (Hori et al., 2010). Therefore,

tegoprazan may be advantageous for rapid symptom relief in GERD treatment.

To determine whether the indications for tegoprazan usage can be expanded to PUD, we

tested the efficacy of tegoprazan in various animal models of PUD. The ED_{50} values of

tegoprazan in NSAID-, ethanol-, and WIRS-induced acute gastric ulcer models were 5–97-

fold more potent than those of esomeprazole. The concentration of tegoprazan was much

higher in the stomach than in the plasma and was present in the gastric tissue even 24 h after

administration in rats. The potent anti-secretory effect of tegoprazan appears to be associated

with its high concentration in the stomach and its rapid onset time. In a previous study, the

effect of tegoprazan on the pH of the gastric perfusate under subcutaneous infusion of 6

ug/kg/h pentagastrin to the gastric fistula of dogs was reported (Takahashi and Take, 2017).

Oral administration of tegoprazan at 3 mg/kg increased the pH from 2 to 4 after 30 min and

then to >6 after 1 h tegoprazan administration. The increase in pH by tegoprazan was

unaffected by subsequent pentagastrin infusion, and the pH was maintained at approximately

7 for 5 h. In contrast, lansoprazole was previously shown to have a significant anti-secretory

effect without cimetidine pretreatment, but this effect disappeared when cimetidine was

administered before lansoprazole (Hori et al., 2011). PPIs could not be transformed to their

active form under neutral conditions resulting from the pretreatment with cimetidine. In our

rat model of sub-chronic acetic acid-induced peptic ulcer, tegoprazan also exhibited superior anti-ulcer activity compared to esomeprazole after 5 days of repeated oral administration. However, PPIs can exhibit maximal activity after treatment with daily doses for 4–5 consecutive days. Combined with the natural healing process, PPI in the sub-chronic model was slightly more potent than in the single administration model. In our PUD model studies, we obtained similar efficacies to that of vonoprazan, which has been used for the treatment of PUD in Japan (data not shown). The potent anti-ulcer activity of tegoprazan shows that it may be effective for patients with peptic ulcer.

In conclusion, tegoprazan is a novel P-CAB that potently and reversibly inhibits gastric H^+/K^+-ATPase. It exhibits superior efficacy in rat models of gastric acid-related diseases compared to esomeprazole. Therefore, tegoprazan is a new therapeutic option that may have enhanced efficacy compared to existing PPI therapies for the treatment of acid-related diseases.

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Authorship Contributions

Participated in research design: Kim DK, Lee SJ, Park CH, Kim BT, Song GS, Moon BS

and Ryu SY

Conducted experiments: Kim DK, Lee KH and Kim Sj

Contributed new reagents or analytic tools: Kim DK and Lee KH

Performed data analysis: Kim DK, Lee KH and Kim SJ

Wrote or contributed to the writing of the manuscript: Kim DK and Ryu SY

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Footnotes

Current affiliation: Not available

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Figure Legends

Fig. 1 Structure of tegoprazan

Fig. 2 *In vitro* inhibitory activities and reversibility of tegoprazan and esomeprazole on porcine gastric H^+/K^+-ATPase. (A) The reaction was initiated by adding tegoprazan and esomeprazole to the reaction buffer followed by incubation at 21℃ for 30min. Data are presented as the mean ± S.E. of 2 independent experiments. (B) The washout method assay was employed to confirm the reversible mechanism of action of tegoprazan. Assays were performed using porcine gastric H^+/K^+-ATPase in freeze-dried vesicles. Data are presented as the mean ± S.D. of 3 independent experiments. Significance of differences between means was determined by the Student's *t-test*. *, $p < 0.05$ versus control.

Fig. 3 Effect of tegoprazan and esomeprazole on gastric acid secretion in pylorus-ligated rats. Tegoprazan and esomeprazole were administered orally 30 min before pylorus ligation. Five hours later, the rats were sacrificed, and the gastric contents were collected and pH was measured. Each column represents the mean ± S.D. from 5–6 rats. Significance of differences between the means was determined by the Mann-Whitney *U* test. *, $p < 0.05$ versus vehicle.

Fig. 4 Effect of tegoprazan and esomeprazole in a rat model of reflux esophagitis. Reflux esophagitis was surgically induced by ligating both the pylorus and foregut of rat stomach.

The rats were laparotomized under anesthesia, and the transitional region between the forestomach and glandular portion, and duodenum were ligated with a silk thread. Prior to duodenum ligation, tegoprazan and esomeprazole were intraduodenally injected. Four hours after surgery, the gastric contents and esophageal mucosa tissues were collected. (A) Macroscopic appearance of the esophageal mucosa in rats. (B) Gastric contents were collected 4 h after surgery, and total acid output was calculated. (C) Esophageal mucosal proteins were extracted from mucosa tissues using lysis buffer, and myeloperoxidase concentration was measured. Each column represents the mean ± S.D. from 3 rats. Significance of the difference between means was determined by the Student's *t-test*. *, $p < 0.05$ versus vehicle.

Fig. 5 Effect of tegoprazan and esomeprazole in rat models of gastric ulcer. Acute gastric ulcers were induced by 3 different inducers. (A) After 30 min of tegoprazan and esomeprazole administration on a single day (closed columns) and daily for 5 days (open columns), naproxen was orally administered to each animal 3 times at 2-h intervals. Four hours after the last naproxen administration, the rats were sacrificed. (B) After 1 h of tegoprazan and esomeprazole administration, absolute ethanol was orally administered for 1 h. (C) Ten minutes after tegoprazan and esomeprazole administration, rats were placed in a restraint cage and immersed in water at 21°C for 4 h. The ulcer area was analyzed by an image analyzer and the ulcer lesion was calculated per total lesion of the stomach. Each

column represents the mean ± S.E. from 6–10 rats. Significance of the difference between

means was determined by the Mann-Whitney U test. *, $p < 0.05$ versus vehicle.

Fig. 6 Effect of tegoprazan and esomeprazole in a rat model of acetic acid-induced gastric

ulcer. The rats were anesthetized with xylazine/ketamine and the stomach was incised via

midline epigastric incision. The antrum was clamped using a sponge forcep and 100 µL of 60 %

acetic acid was then injected into the clamped area for 40 s. After 2 days, tegoprazan and

esomeprazole were orally administered for 5 days. (A) Gastric ulcer area data are presented

as the mean ± S.D. from 10 rats. Significance of the difference between means was

determined by the Mann-Whitney U test. *, $p < 0.05$ versus vehicle. (B) Gross appearance of

gastric mucosa in rats and quantitative analysis.

Fig. 7 Concentration-time profiles of tegoprazan in plasma and the stomach after oral

administration of tegoprazan at a dose of 10 mg/kg in rats. Plasma and stomach samples were

obtained 0.25, 0.5, 1, 6, 12, and 24 h after dosing. Plasma and gastric concentration of

tegoprazan are shown in ng/mL and ng/g, respectively. Each point represents the mean ± S.D.

of five rats.

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Tables

TABLE 1

ED$_{50}$ values of tegoprazan and esomeprazole in reflux oesophagitis model

GERD model	ED$_{50}$ (mg/kg)	
	Tegoprazan (CI)	Esomeprazole (CI)
Total acid output	2.0 (0.9-4.3)	32.5 (14.3-73.8)
MPO	5.9 (3.0-11.8)	29.2 (13.2-64.7)

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TABLE 2

ED$_{50}$ values of tegoprazan and esomeprazole in peptic ulcer models

PUD models	ED$_{50}$ (mg/kg)	
	Tegoprazan	Esomeprazole
Naproxen (Day 1)	0.1	3.9
Naproxen (Day 5)	0.1	0.9
Ethanol (Day 1)	1.4	7.3
WIRS (Day 1)	0.1	9.7

Figures

Fig. 1

Fig. 2

A

B

Fig. 3

A

B

Fig. 4

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Fig. 5

A

B

C

Fig. 6

Fig. 7