KFP-H008 blocks gastric acid secretion through inhibiting H⁺-K⁺-ATPase

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ABSTRACT

1-(5-(1H-indol-5-yl)-1-(pyridin-3-ylsulfonyl)-1H-pyrrol-3-yl)-N-methylmethanamine (KFP-H008), a novel and potent potassium-competitive acid blocker for the treatment of acid secretion related diseases, has not been reported previously. In this study, we demonstrated that KFP-H008 inhibits basal acid secretion, 2-deoxy-D-glucose (2DG)-stimulated gastric acid secretion in rats. KFP-H008 blocked histamine-stimulated acid secretion in rats and heidenhain pouch dogs and reversed acid output in isolated gastric perfusion under histamine stimulation. In all the animal experiments, KFP-H008 exerted a more effective, potent and longer-lasting inhibitory action in comparison with lansoprazole, a proton pump inhibitor (PPI) commonly used in clinic. KFP-H008 inhibited H⁺-K⁺-ATPase activity both at pH 6.5 and pH 7.5, and was unaffected by pH. The inhibitory action was reversible and was achieved in a K⁺-competitive manner. Furthermore, KFP-H008 did not affect Na⁺-K⁺-ATPase activity, thus exhibiting high selectivity, which is different from PPIs. In all, KFP-H008, a novel potassium-competitive acid blocker, may provide new option for the patients with acid-related diseases and provide longer-lasting inhibitory action than drugs commonly used in clinical treatment.

1. Introduction

Acid-related diseases (ARD) refer to gastrointestinal diseases caused by excessive gastric acid secretion or gastric acid sensitivity, which mainly include reflux esophagitis, gastric ulcer, dyspepsia, gastritis, duodenum ulcers, Zollinger-Ellison syndrome and so on (Shin and Sachs, 2009). These diseases seriously affect the quality of patients’ daily life, and the incidence increased year by year. Currently, the treatment for digestive tract diseases in clinic is to control the secretion of gastric acid, the relevant drugs include acid neutralizing agents, gastrin receptor antagonist, cholinergic receptor antagonist, histamine H₂ receptor antagonist as well as proton pump inhibitor (PPIs).

PPIs block gastric acid secretion through irreversibly inhibiting H⁺-K⁺-ATPase activity by binding to the sulfhydryl group of enzyme (Arison, 2016). The PKa of PPIs is about 4.0–5.0, an inactive state in which PPIs are not easy to dissociate. PPIs have potent inhibitory activity against acid secretion, while a lot of limitations have been acknowledged such as the dependence on CYP450 enzymes, which leads to significant individual differences (Kagami et al., 2016; Kondo et al., 2012). PPIs therapy frequently does not inhibit acid secretion throughout a 24-h period because of a short plasma half-life (t₁/₂) (Arikawa et al., 2012). PPIs need to take 4–5 days to achieve maximal acid inhibitory activity at therapeutic dose, as PPIs only choose to bind to the activated H⁺-K⁺-ATPase under acidic condition (Shin et al., 2011).

A new class of acid suppressants, known as potassium-competitive acid blocker (P-CABs), exhibited reversible and long-term inhibitory activities against gastric H⁺-K⁺-ATPase (Äbelö et al., 2006). P-CABs can stay at low pH and inhibit gastric H⁺-K⁺-ATPase in a K⁺-competitive manner (Inatomi et al., 2016). Animal experiments and clinical studies have demonstrated that the oral dose of P-CABs are in a linear relationship with plasma concentration, so it comes into effect rapidly, which is able to achieve the maximum therapeutic effect within 1 h (Otake et al., 2016). Since the metabolism of P-CABs is independent of CYP2C19, the individual difference in drug efficacy is very small. Many P-CABs have been reported such as SCH28080 ((3-(cyanomethyl)-2-methyl-8-(phenylmethoxy) imidazo[1,2-a]-pyridine)) (Li et al., 2013), Revaprazan (5, 6-dimethyl-2-(4-fluorophenylamino)-4-(1-methyl-1, 2, 3, 4-tetrahydrosiquinoline-2-yl)pyrimidine) (Lee et al., 2012; Yenisehirli and Onur, 2006), PF-03716556 (N-(2-Hydroxyethyl)-N, 2-dimethyl-8-(((4R)-5-methyl-3,4-dihydro-2H-chromen-4-yl)amino)imidazo[1, 2-alpyridine-6-carboxamide) (Mori et al., 2009), TAK-438 (1-{5-(2-fluorophenyl)-1-(pyridin-3-ylsulfonyl)-1H-

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http://dx.doi.org/10.1016/j.ejphar.2017.06.020
Received 27 February 2017; Received in revised form 14 June 2017; Accepted 15 June 2017
Available online 16 June 2017
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pyrrol-3-yl-N-methylmethanamine monofumarate) (Otake et al., 2016). P-CABs provide a new choice for patients with intractable ARD. Due to the hepatic toxicity or insufficient efficacy, most P-CABs are not being used clinically except Revaprazan and TAK-438 (Bi et al., 2014; Luo et al., 2014).

We have discovered a novel P-CAB, 1-(5-(1H-indol-5-yl)-1-(pyridin-3-ylsulfonyl)-1H-pyrrol-3-yl)-N-methylmethanamine (KFP-H008, M: 366.11). In this study, we investigated its effects on basal gastric acid secretion, 2-deoxy-D-glucose-stimulated acid secretion in rats, effects on histamine-stimulated acid secretion in rats and heidenhain pouch dogs, in comparison with TAK-438 and lansoprazole (Campbell et al., 2008; Debnath et al., 1974).

2. Materials and methods

2.1. Chemicals and reagents

KFP-H008 and TAK-438 were provided by Jiangsu Carefree Pharmaceutical Co., Ltd. (Nanjing, China). Lansoprazole (99.45% purity) was purchased from Yuancheng Gongchuang Technology Co., Ltd. (Wuhan, China). In the in vivo experiments, KFP-H008 and lansoprazole were dissolved in deionized water and administered orally. When measuring H⁺-K⁺-ATPase and Na⁺-K⁺-ATPase activity in vitro, KFP-H008, lansoprazole and TAK-438 were dissolved in dimethyl sulfoxide. 2-Deoxy-D-glucose (2-DG) was purchased from Sigma-Aldrich Co. LLC. (USA), dissolved in deionized water before use. Drugs and vehicle were given orally to rats before anesthesia (chloral hydrate 38–42 g were randomly divided into six groups: sham, model, KFP-H008 (1 mg/kg, 2 mg/kg, and 4 mg/kg), lansoprazole (2 mg/kg), TAK-438 (2 mg/kg). Sham group and model group were treated with deionized water. Drugs and vehicle were given orally to rats before anesthesia. 1 h after administration, pylorus was ligated. 5 min later, 2DG was injected subcutaneously. At 3 h after 2DG administration, the rats were killed by CO₂ asphyxiation. The gastric contents were collected in a 10 ml-centrifuge tube after removing their stomachs. Centrifuging at 1500 g for 10 min, and then determining the total acid output during 3-h period by 0.1 M NaOH (Maeda-Hagiwara and Watanabe, 1983). % Inhibition = (average of model acid – drug acid)/ average of model acid × 100%.

2.2. Animals

All the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Ethics Committee of China Pharmaceutical University. Male and female (half by half) Sprague-Dawley rats weighing 180–220 g were purchased from Experimental Animal Center of Zhejiang Province, Production License No. SCXK (Zhe) 2014-0001. Male Sprague-Dawley rats weighing 38–42 g were purchased from Experimental Animal Center of Zhejiang Province, Production License No. SCXK (Zhe) 2014-0001. Male Beagle dogs weighing 10.1–12.5 kg were bought from Jiangian Biotechnology Co., Ltd. (Shanghai, China), Production License No. SCXK (Hu) 2015-0005. Rabbits weighing 1.8–2.0 kg were purchased from Qinglongshan Animal Farm of Nanjing, China, Production License No. SCXK (Su) 2012-0008. All animals in this study were housed under a 12 h light/12 h dark cycle at controlled temperature (22–26 °C) and humidity (40–70%), with free access to food and water. The rats were fasted for 24 h before the experiment but given free access to water. A gastric pouch was constructed in male Beagle dogs before the Heidenhain pouch dog experiment. Heidenhain pouch dogs were fasted for 14 h before the experiment but given free access to water.

2.3. Measurement of gastric acid secretion

2.3.1. Gastric acid secretion in pylorus-ligated rats

Rats were randomly divided into six groups: vehicle, KFP-H008 (1 mg/kg, 2 mg/kg, and 4 mg/kg), lansoprazole (2 mg/kg), TAK-438 (2 mg/kg). Vehicle group was treated with deionized water. Drugs and vehicle were given orally to rats before anesthesia (chloral hydrate 300 mg/10 ml/kg). 1 h after administration, pylorus was ligated. At 3 h after the pyloric ligation, the rats were killed by CO₂ asphyxiation. The gastric contents were collected in a 10 ml-centrifuge tube after removing their stomachs. Centrifuging at 1500 g for 10 min, and then determining the total acid output during 3-h period by 0.1 M NaOH (Campbell et al., 2008; Debnath et al., 1974).

2.3.2. 2DG-stimulated acid secretion in anesthetized rats

Rats were randomly divided into seven groups: sham, model, KFP-H008 (1 mg/kg, 2 mg/kg, and 4 mg/kg), lansoprazole (2 mg/kg), TAK-438 (2 mg/kg). Sham group and model group were treated with deionized water. Drugs and vehicle were given orally to rats before anesthesia. 1 h after administration, pylorus was ligated. 5 min later, 2DG was injected subcutaneously. At 3 h after 2DG administration, the rats were killed by CO₂ asphyxiation. The gastric contents were collected in a 10 ml-centrifuge tube after removing their stomachs. Centrifuging at 1500 g for 10 min, and then determining the total acid output during 3-h period by 0.1 M NaOH (Maeda-Hagiwara and Watanabe, 1983). % Inhibition = (average of model acid – drug acid)/ average of model acid × 100%.

2.3.3. Histamine-stimulated acid secretion in anesthetized rats

The histamine-stimulated acid secretion in anesthetized rats was measured as described for 2DG-stimulated acid secretion, with the exception that 5 min after pylorus ligation, histamine 2HCl (30 mg/10 ml/kg) was injected subcutaneously (Bastaki et al., 1995).

2.3.4. Measurement of the acid output of isolated gastric perfusion under histamine stimulation

Male rats weighing 38–42 g were randomly divided into six groups: sham, KFP-H008 (1 mg/kg, 2 mg/kg, and 4 mg/kg), lansoprazole (2 mg/kg), TAK-438 (2 mg/kg). Open the abdominal cavity after the drug administration (Bunce and Parsons, 1976; Hills et al., 1996; Welsh et al., 1993; Komazaka et al., 2002; Kowalewski and Kolodej, 1975).

2.3.5. Histamine-stimulated acid secretion in heidenhain pouch dogs

Before the heidenhain pouch dog experiment, a gastric pouch was constructed according to the Heidenhain method in male Beagle dogs. After anesthetizing with 3% pentobarbital sodium (30 mg/kg) intravenously in the forelimb, the dog’s stomach was exposed, choose 2 points in the greater curvature, ligating the blood vessels and cutting the anterior wall of stomach into two parts with a main body of the stomach and a pouch with adequate blood supply. The pouch was drained into an implanted metal cannula. Then closing the pouch and brought the cannula out of the abdominal cavity. After recovering from
surgery, the Heidenhain pouch dogs were randomly divided into six groups: model, KFP-H008 (0.1 mg/kg, 0.3 mg/kg, and 1 mg/kg), lansoprazole (0.3 mg/kg) group and TAK-438 (0.3 mg/kg). Animals were administered orally with drugs and vehicle. Histamine 2HCl (30 μg/kg) was injected subcutaneously a day before and 1, 3, 6, 24, 48 h after drug administration. Acid output during 90-min period was collected from the pouch, the total acid output during 90-min period at each time point was determined by 0.1 M NaOH, and expressed as a percentage of the pre-dosing value measured 1 day before the drug administration. The area under curve of different group from 0 to 25.5 h after dosing was also calculated to evaluate the inhibition effect (Branden et al., 2010; Uchiyama et al., 1999).

2.4. Preparation of rabbit gastric H⁺-K⁺-ATPase

The fresh rabbit fundus mucosa was isolated, washed with tap water and 3 M NaCl solution. The homogenizing buffer (4 ml/g) was added and homogenized for 2–5 min. The homogenate was centrifuged at 20,000g for 30 min at 4 °C, and the supernatants were re-centrifuged at 100,000g for 90 min at 4 °C. The precipitate was collected and suspended in the homogenizing buffer (containing 1 mM EDTA, 10 mM Tris-HCl pH 6.8, 0.25 mM sucrose), then layered over 7.5% Ficoll (w/w) solution in the homogenizing buffer. The homogenate was centrifuged at 100,000g for 60 min; the middle layer was collected and diluted with homogenizing buffer, then centrifuged at 100,000g for 90 min. The precipitate was suspended in homogenizing buffer to give a concentration of 0.5 mg/ml. The final suspension was stored at −80 °C until use (Kubo et al., 1995; Nagaya et al., 1989). Protein concentration was determined by total protein quantitative assay kit (Nanjing Jiancheng Bioengineering Institute. Nanjing, China).

2.5. Measurement of H⁺-K⁺-ATPase activity

The ATPase activity was determined by measuring the inorganic phosphate released by the hydrolysis of ATP (Fiske and Subbarow, 1925; Im et al., 1985). Protein (0.25 μg) was preincubated at 37 °C for 30 min in 90 μl of 50 mM HEPES-Tris buffer (pH 6.5 or 7.5), which contained 5 mM MgCl₂, 10 μM valinomycin, 0 or 5 mM KCl and different concentrations of KFP-H008, TAK-438, lansoprazole. The reaction was initiated by adding 10 μl of 5 mM ATP 2Na and the preparation was incubated for 30 min at 37 °C. The reaction was halted by the addition of 30 μl of malachite green reagent. The ATPase activity was calculated as described above.

Measurement of enzyme kinetics was determined in the presence of different concentrations of KCl. Enzyme mixtures containing KCl and different drugs of various concentrations were incubated for 30 min at 37 °C. The ATPase activity was calculated as described above (Hori et al., 2010).

2.6. Measurement of Na⁺-K⁺-ATPase activity

Porcine cerebral cortex Na⁺-K⁺-ATPase activity was measured as described as H⁺-K⁺-ATPase, with the exception that the 90 μl of reaction mixture was composed of 0.05 unit Na⁺-K⁺-ATPase, 50 mM HEPES-Tris (pH 7.5), with or without 100 mM NaCl or 10 mM KCl, 2 mM MgCl₂ and the test compound (Fujii et al., 2007; Nishida et al., 2012).

2.7. Statistical analysis

All data in the study were represented as mean ± S.E.M., calculated by Graphpad Prism 5.0. In the gastric secretion experiment, at least six independent preparations were performed. In the measurement of H⁺-K⁺-ATPase activity and Na⁺-K⁺-ATPase activity, at least three independent operations were repeated. Statistical comparisons were made by Paired-Samples T Test (for two-group comparisons) and one-way ANOVA (for multiple group comparisons) using SPSS (IBM SPSS Statistics v19.0). P Values < 0.05 was considered statistically significant.

3. Results

3.1. KFP-H008 significantly decreases basal gastric acid secretion

The total acid outputs during 3-h period were measured. KFP-H008 at doses of 1, 2, 4 mg/kg and TAK-438 at 2 mg/kg apparently inhibited reverse the action.

In another experiment, the reversibility of the inhibitory effects of drugs was measured after a dilution procedure (Hori et al., 2010). 2 μl compound solution added in 12 μl of 0.002% HCl solution was preincubated for 5 min and then 4 μl protein was added in. The preparation was incubated for 5 min at room temperature. 2 μl of 500 mM HEPES-Tris buffer (pH 6.5) was added to the preparation as a normalization procedure. Then 2.2 μl of the mixture above was diluted with 78 μl buffer containing 50 mM HEPES-Tris (pH 6.5), 12.5 μM valinomycin and 5 mM MgCl₂. 9.8 μl of the compound was added as the undiluted control, while 9.8 μl of vehicle was added as the dilution treatment group. 60 min later, the reaction was initiated in the presence of 10 μl of 5 mM ATP and incubated for 60 min at 37 °C. The reaction was halted by the addition of 30 μl of malachite green reagent. The ATPase activity was calculated as described above.
basal gastric acid secretion versus the vehicle group (P < 0.01) in a dose-dependent manner (Fig. 1A). The basal acid output of vehicle group was about 78 μmol/3 h. Lansoprazole (2 mg/kg) did not show any significant inhibitory effect on basal acid secretion, and KFP-H008 inhibited basal acid output apparently in comparison with the lansoprazole group at the same dosage of 2 mg/kg (Fig. 1A). In the isolated gastric perfusion experiment, after administration of KFP-H008, lansoprazole and TAK-438 at various dosage, the basal acid output of isolated gastric perfusion of each group was decreased significantly (P < 0.01, Fig. 1B). KFP-H008 at the dose of 2 mg/kg exhibited more effects on the inhibitory activities against the basal acid output in comparison with lansoprazole group in isolated gastric perfusion (P < 0.01, Fig. 1B).

3.2. KFP-H008 inhibits 2DG-stimulated gastric acid secretion in rats

After stimulated with 2DG, the acid output in model group was about 452 μmol/3 h in comparison with the sham group of 79 μmol/3 h, which means we succeeded conducting the model. KFP-H008 at doses of 2, 4 mg/kg, lansoprazole (2 mg/kg) and TAK-438 (2 mg/kg) showed strong inhibitory effects on 2DG-stimulated acid secretion in comparison with the model group (P < 0.05, Fig. 2A). Furthermore, the inhibitory activities against acid secretion of KFP-H008 were presented in a dose-dependent manner (Fig. 2A), and the % inhibition at the dose of 4 mg/kg was the highest (61.36%, Fig. 2B).

3.3. KFP-H008 decreases histamine-stimulated gastric acid secretion

In the pylorus-ligated rats, when gastric aid secretion was stimulated with histamine, the acid output of the model group was up to 166 μmol/3 h in comparison with the sham group of 72 μmol/3 h. All of the drugs presented remarkable inhibition (P < 0.01), and KFP-H008 at doses of 1, 2, 4 mg/kg inhibited histamine-stimulated acid output in a dose-independent manner (Fig. 3A). The % inhibition of KFP-H008 at doses of 4, 2, 1 mg/kg, lansoprazole (2 mg/kg) and TAK-438 (2 mg/kg) were 82.84%, 75.08%, 66.95%, 60.69% and 86.03%. (Fig. 3B). In the isolated gastric perfusion experiment, KFP-H008 and lansoprazole both showed inhibitory effects on gastric acid secretion after stimulation of histamine compared to the model group, while KFP-H008 at 4 mg/kg presented more potent and longer-lasting inhibitory action than any other dosages or lansoprazole, as even the concentration of histamine up to 1 mM, KFP-H008 still inhibited the acid secretion completely (92.93% pre-dosing) and the quantity of acid output rose slowly (Fig. 3C). In heidenhain pouch dogs’ experiment, KFP-H008 at doses of 0.1, 0.3, 1 mg/kg exhibited inhibitory action on histamine-stimulated gastric acid secretion in a dose-dependent manner, and the inhibitory effect lasted for more than 48 h (Fig. 3D). KFP-H008 at doses of 0.1–1 mg/kg showed a statistically significant inhibitory effect on gastric acid secretion 3 h after administration in comparison with 0.3 mg/kg lansoprazole (P < 0.01). 24 h and 48 h after administration, 0.3 mg/kg of KFP-H008 exhibited more potent inhibitory action on gastric acid secretion than 0.3 mg/kg of lansoprazole (P < 0.05, Fig. 3D).

3.4. KFP-H008 inhibits rabbit gastric H+-K+-ATPase activity both at pH 6.5 and pH 7.5

In order to verify whether the pH condition affect the inhibitory activity of KFP-H008 against H+-K+-ATPase, different acidic conditions were constructed. Under pH 6.5 condition, all of the three compounds (KFP-H008, TAK-438 and lansoprazole) exhibited inhibitory activities against H+-K+-ATPase in a concentration-independent manner (Fig. 4A, B, C). The inhibitory effects of KFP-H008 and TAK-438 were similar. The IC50 values of KFP-H008, TAK-438, and lansoprazole were 0.029, 0.02, 7.51 μM, respectively (Fig. 4D). Under another neutral condition, pH 7.5, the inhibitory action of KFP-H008 and TAK-438 seems never change, while the inhibitory activity of lansoprazole was weaker under pH 7.5 (Fig. 4).

3.5. KFP-H008 has no inhibitory effect on Na+-K+-ATPase activity

To determine the selective inhibitory activities of the three compounds, the inhibitory effects on Na+-K+-ATPase were studied. The data showed that even at concentrations 500 times higher than their IC50 values against H+-K+-ATPase, neither KFP-H008 nor TAK-438 exhibited inhibitory activity against Na+-K+-ATPase, while lansoprazole at 100 μM (which was only about 13 times higher than the IC50 value of lansoprazole against H+-K+-ATPase at pH 6.5) inhibited Na+-K+-ATPase slightly (27.4%, Table 1).

3.6. The inhibitory activity of KFP-H008 against H+-K+-ATPase is not affected by DTT

Sulfhydryl-containing compound DTT at low concentrations will protect H+-K+-ATPase against PPIs as PPIs work by binding to the sulfhydryl groups of the enzyme. In this study, we found that the inhibitory activities of KFP-H008 (0.1 μM) and TAK-438 (0.1 μM) were not affected by the presence of 100 μM DTT, while the activity of lansoprazole (100 μM) were reversed significantly by 100 μM DTT (P < 0.01, Fig. 5). Each concentration of the three compounds we chose was at a submaximal inhibitory concentration.

3.7. A dilution procedure reverses the inhibitory activities of KFP-H008 against H+-K+-ATPase

To investigate the reversibility of the inhibitory effect of KFP-H008,
we examined the effect of dilution on the inhibitory activities of the three compounds. As a result, the inhibitory activities of KFP-H008 and TAK-438 at the submaximal concentration were remarkably reversed by diluting the reaction mixture (Fig. 6). The % inhibition values of KFP-H008 (0.1 μM), TAK-438 (0.1 μM), lansoprazole (100 μM) without dilution were 80.16%, 73.6%, 86.48%, and the values after dilution were 16.14%, 15.28%, 90.64%, respectively. The data also represented that effect of lansoprazole at the submaximal inhibitory concentration was not affected by dilution (Fig. 6).

3.8. The inhibitory activity of KFP-H008 against H⁺-K⁺-ATPase is in a K⁺-competitive manner

Kinetic experiments for KFP-H008 and TAK-438 were performed

Fig. 3. Effects of KFP-H008 on histamine-stimulated gastric acid secretion. (A, B) Effects were tested in anesthetized rats. The vehicle, KFP-H008, lansoprazole and TAK-438 were administrated orally (1 ml/100 g) 1 h before pylorus ligation and histamine (30 mg/10 ml/kg s.c.) administration. 3 h later, the gastric contents were collected, and acid output in 3 h was calculated. n = 10. (B) % Inhibition = (average of model_acid – drug_acid)/ average of model_acid x100%. (C) Effects were tested in isolated gastric perfusion under histamine stimulation. The acid output during the 30-min period from each point was expressed as a percentage of the pre-dosing value measured before administration of histamine. n = 6. (D) Effects were tested in histamine-stimulated Heidenhain pouch dogs. The vehicle, KFP-H008, lansoprazole and TAK-438 were administrated orally. Histamine HCl was injected (s.c.) a day before and 1, 3, 6, 24, 48 h after vehicle and drugs administration. The acid output during the 90-min period from each point was calculated and expressed as a percentage of the pre-dosing value measured 1 day before administration. n = 8. Each column and point represents the mean ± S.E.M. ▲▲, P < 0.01 versus sham group. **, P < 0.01 versus model group.

Fig. 4. Inhibitory activities of KFP-H008 against gastric H⁺-K⁺-ATPase in rabbits. The enzyme was incubated for 30 min with various concentrations of KFP-H008 (A), TAK-438 (B) and lansoprazole (C) at pH 6.5 and pH 7.5. (D) IC₅₀ values of KFP-H008, TAK-438, lansoprazole against gastric H⁺-K⁺-ATPase were analyzed by Graphpad Prism 5.0. Each value represents mean ± S.E.M. of 3 independent experiments.
The inhibitory action of each compound on gastric H+-K+-ATPase was measured in the H+-K+-ATPase activity in a K+-competitive manner (Fig. 7) and the results demonstrated that both KFP-H008 and TAK-438 inhibited the activity of H+-K+-ATPase. The Ki values of KFP-H008 and TAK-438 were calculated to be 0.004 and 0.003 μM, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Concentration/Compounds</th>
<th>μM</th>
<th>n</th>
<th>Inhibition (%)</th>
<th>Mean ± S.E.M.</th>
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<tr>
<td>KFP-H008</td>
<td>10</td>
<td>3</td>
<td>2.75 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>TAK-438</td>
<td>10</td>
<td>3</td>
<td>3.2 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Lansoprazole</td>
<td>100</td>
<td>3</td>
<td>27.4 ± 1.64</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 5.** Effect of DTT on the inhibitory activities of KFP-H008 against H+-K'-ATPase. The inhibitory action of each compound on gastric H’-K’-ATPase was measured in the presence or absence of 100 μM DTT. Each column represents the mean ± S.E.M. of 5 independent experiments. **, P < 0.01 versus the group in the absence of 100 μM DTT.

**Fig. 6.** Effects of dilution on the inhibitory activities of KFP-H008 against H’-K’-ATPase. The inhibitory action of each compound on gastric H’-K’-ATPase was measured 60 min after dilution or without dilution of the mixture. Each column represents the mean ± S.E.M. of 3 independent experiments. **, P < 0.01 versus the group without diluting.

to confirm mode of inhibition of the gastric H’-K’-ATPase. Lineweaver-Burk analysis of KFP-H008 and TAK-438 were conducted. The results demonstrated that both KFP-H008 and TAK-438 inhibited H’-K’-ATPase activity in a K’-competitive manner (Fig. 7) and the inhibition was reversible. The Ki values of KFP-H008 and TAK-438 we calculated were 0.004 and 0.003 μM, respectively.

### 4. Discussion

KFP-H008, a potent P-CAB, has not been reported previously. In this study, we first reported that KFP-H008 inhibits gastric acid secretion in normal or pathological animals. The underlying mechanism of this antisecretory effects is related to H’-K’-ATPase inhibition.

KFP-H008 exhibited more potent effect on basal gastric acid in pylorus-ligated rats as well as in isolated gastric perfusion, in comparison with lansoprazole, a typical PPI. It has been reported that administration of lansoprazole does not completely inhibit basal gastric acid secretion, because PPIs themselves do not react with SH groups of H’+K’-ATPase (a transmembrane enzyme present in parietal cells, the target molecule for PPIs), they transform into various structures and undergo molecular rearrangement under acidic conditions in parietal cells that do react with SH groups, and then come into effect (Abe et al., 2011; Mossner, 2016; Savarino et al., 2017). In our study, lansoprazole showed no significant effect on basal acid secretion in pylorus-ligated rats versus the sham group (P > 0.05), while showed obvious antisecretory effects on basal isolated gastric perfusion. The reason for this phenomenon may due to the difference of the time after administration, or the different conditions in vivo and in vitro cause changed sensibilities of drugs. KFP-H008 and TAK-438 exerted their antisecretory effect without the activation of H’+K’-ATPase while effect of PPIs such as lansoprazole is required for its activation under acidic condition.

2-deoxy-D-glucose (2DG) is capable to stimulate the gastric acid secretion, and was widely used in acid secretory model in rats (Hori et al., 2010; Maeda-Hagiwara and Watanabe, 1983). In our experiments, KFP-H008 at doses of 2, 4 mg/kg showed strong inhibitory effects on 2DG-stimulated acid secretion in a dose-dependent manner.

Histamine 2HCl is an active amine compound. As a chemical substance in the body, histamine is able to affect the response of many cells, leading to allergies, inflammation, and gastric acid secretion (Kowalewski and Kolodej, 1975). In our study, we explored the inhibitory effects of KFP-H008 on histamine-stimulated gastric acid secretion in pylorus-ligated rats, isolated gastric perfusion and heidenhain pouch dogs. In the pylorus-ligated rats, all of the drugs presented remarkable inhibition (P < 0.01). In the isolated gastric perfusion experiment, KFP-H008 at dose of 4 mg/kg was capable to inhibit the acid secretion completely despite the concentration of histamine upping to 1 mM, the duration of the inhibitory effect seems long. In heidenhain pouch dogs’ experiments, we found that KFP-H008 at dose of 1 mg/kg inhibited gastric acid secretion by 60% within 3 h, and the inhibitory effect of KFP-H008 lasted for more than 48 h. These results suggest that both in rats and heidenhain pouch dogs, KFP-H008 exhibited stronger and longer-lasting antisecretory effects, as well as a rapid onset of action than lansoprazole. It is reported that in clinic, PPIs are used commonly in reflux esophagitis, the symptom relief are
associated with gastric pH of > 4 holding time, therefore, the duration of gastric pH > 4 holding time is important for the treatment of reflux esophagitis (Hori et al., 2011; Konturek et al., 2013; Maradey-Romero and Fass, 2014), while PPIs cannot inhibit night-time gastric acid secretion completely despite the administration time is before dinner, because the duration of acid suppression is time-limited (Mossner, 2016; Zhi-Cheng et al., 2016). PPIs usually require repeated administration over several days to reach a stable, maximal action at therapeutic doses in humans (Matsukawa et al., 2011; Mori et al., 2009). But the duration of the antisecretory effect of P-CABs is much longer than their plasma half-life due to the covalent binding (Kagami et al., 2016). KFP-H008 may bind to H¹⁻K⁺-ATPase tightly so the inhibitory effect of KFP-H008 lasted for more than 48 h in heidenhain pouch dogs.

KFP-H008 is one of the pyrrole-sulfonyl-derivatives, the structure is partly similar to TAK438, a novel P-CAB. Thus, the underlying mechanism of KFP-H008 may be similar to P-CABs. To elucidate the exact mechanism underlying the antisecretory effect, the inhibitory effect of KFP-H008 on H¹⁻K⁺-ATPase was evaluated. In our mechan-ism experiments, KFP-H008 showed a more potent inhibitory action on gastric H¹⁻K⁺-ATPase activity than lansoprazole, the inhibitory effect of KFP-H008 on H¹⁻K⁺-ATPase at pH 6.5 was about 250 times stronger than that of lansoprazole, which is consistent with the observed potency in vivo. The data we obtained showed the similar effect as TAK-438 (Scott et al., 2015), a novel P-CAB not widely used in clinic, developed by Takeda Pharmaceutical Company Limited, Osaka, Japan. It has been reported that TAK-438 was a P-CAB with more potent efficacy and longer-lasting effect on gastric acid inhibition than even PPIs and the aforementioned P-CABs (Akazawa et al., 2016; Ashida et al., 2016; Maruoka et al., 2016; Shin et al., 2011), so we choose TAK-438 as another positive P-CAB control in this study. We found that the inhibitory of KFP-H008 and TAK-438 was not affected by ambient pH, while the inhibition of lansoprazole was attenuated at pH 7.5, from 7.51 μM at pH 6.5–6.95 μM at pH 7.5. Acidic condition in parietal cells is the key requirement for P-CABs to rearrange their structure and bind to the transmembrane domains (SH-domains) of H¹⁻K⁺-ATPase (Nagaya et al., 1989). Therefore, the inhibitory activity of lansoprazole decreases under the neutral condition.

Since the inhibitory effect of PPIs against H¹⁻K⁺-ATPase activity can usually be blocked by a thiol reagent such as DTT (Nagaya et al., 1989; Paresi et al., 2016), DTT is able to bind to the PPIs then interfere with the binding between PPI and H¹⁻K⁺-ATPase. In our study, the inhibitory actions of KFP-H008 and TAK-438 against H¹⁻K⁺-ATPase were unaffected by the presence of 100 μM DTT, in contrast to KFP-H008 or TAK-438, the inhibitory effect of lansoprazole was blocked seriously with the treatment of 100 μM DTT.

In another experiment, a dilution procedure was constructed, the inhibitory activities of KFP-H008 and TAK-438 were reversed, whereas that of lansoprazole represented the same result as diluted before, indicating PPIs bind to gastric H¹⁻K⁺-ATPase irreversibly. As a result of analysis, KFP-H008 and TAK-438 demonstrated that KFP-H008 is a P-CAB de-termined gastric H¹⁻K⁺-ATPase (Nagaya et al., 1989). Therefore, the inhibitory activity of lansoprazole decreases under the neutral condition.

The amino acid sequences of H¹⁻K⁺-ATPase and Na⁺⁻K⁺-ATPase are high homologous, PPIs usually have the ability to inhibit Na⁺⁻K⁺-ATPase slightly (Hori et al., 2010; Mori et al., 2009; Yenisehirli and Onur, 2006). Finally, we carried out an experiment to explore the selectivity of KFP-H008. In our study, KFP-H008 and TAK-438 did not inhibit Na⁺⁻K⁺-ATPase activity, even at the concentration of 10 μM, 500 times higher than their IC50 values against H¹⁻K⁺-ATPase. And lansoprazole exhibited inhibitory activity against Na⁺⁻K⁺-ATPase at 100 μM, which concentration was only about 13 times higher than the IC50 value of lansoprazole against H¹⁻K⁺-ATPase at pH 6.5. These results reflect the selectivity of KFP-H008. On the other hand, there is evidence demonstrate that stomach is the only organ in humans expressing significant levels of the P-CAB target H¹⁻K⁺-ATPase (Herrmann et al., 2007), further indicating the high selectivity of P-CABs.

In summary, KFP-H008 showed a more potent and longer-lasting effect on gastric acid secretion than lansoprazole in rats and heidenhain pouch dogs in vivo. In vitro, KFP-H008 demonstrated a potent inhibitory effect on gastric H¹⁻K⁺-ATPase in a reversible, selective and K⁺-competitive manner no matter the gastric secretory state. The potency of KFP-H008 and TAK-438 seems similar from the results obtained currently, and the differences between them in other research areas are required to be investigated intensively in the future, whereas KFP-H008 exhibit more excellence and advantages in antisecretory effect than clinically commonly used PPIs such as lansoprazole, and is superior to lansoprazole in the underlying mechanism study. The present data demonstrated that KFP-H008 is a novel and potential antisecretory drug that would provide an improvement over the current PPIs-based treatment and provide significant benefit to the patients with acid-related disease that do not adequately respond to PPIs.

Acknowledgments

Financial support for this study was provided by Qinglan Project (2016) and National Major Scientific and Technological Special Project for “Significant New Drugs Development” during the Thirteenth Five-year Plan Period (No. 2016ZX09101031).

References

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