Studies on the Mechanism of Action of the Gastric H^+,K^+ -ATPase Inhibitor SPI-447

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ABSTRACT—3-Amino-5-methyl-2(2-methyl-3-thienyl)-imidazo[1,2-*a*]thieno[3,2-*c*]pyridine, SPI-447, is a potent gastric H^+,K^+ -ATPase inhibitor, but a detailed mechanism of the inhibition is unknown. This study was designed to investigate the mechanism by which SPI-447 inhibits gastric H^+,K^+ -ATPase. For this purpose, the inhibitory action of SPI-447 on gastric H^+,K^+ -ATPase from porcine gastric mucosa was compared with that of omeprazole (an irreversible inhibitor) and SCH28080 (a reversible inhibitor). All compounds produced dose-dependent inhibition of gastric H^+,K^+ -ATPase, and the inhibitory intensities were increased under acidic conditions. The anti- H^+,K^+ -ATPase actions of SPI-447 and SCH28080 were attenuated by dilution, but not influenced by glutathione pretreatment. In contrast, that of omeprazole was not influenced by dilution, but was suppressed by glutathione pretreatment. KCl addition reversed the inhibition of H^+,K^+ -ATPase-mediated H^+ -transport by SPI-447 and SCH28080, but had no effect on that by omeprazole. The anti-gastric H^+,K^+ -ATPase action of SPI-447 was additive with that of SCH28080. SPI-447 and SCH28080 had no effect on Na^+,K^+ -ATPase activity. These findings indicated that the inhibitory mechanism of SPI-447 on gastric H^+,K^+ -ATPase was similar to that of SCH28080, but different from that of omeprazole; i.e., 1) reversible, 2) SH-group independent, 3) K^+ -competitive, and 4) highly specific against gastric H^+,K^+ -ATPase.

Keywords: H^+,K^+ -ATPase inhibitor, Anti-ulcer drug, SPI-447, Omeprazole, SCH28080

Inhibition of the final step of acid secretion would be the most effective way of controlling acid secretion in the stomach. There are two classes of proton pump inhibitors that have this effect. The covalent class consists of the pyridyl methyl sulfanyl benzimidazoles, such as omeprazole (1, 2), lansoprazole (3), pantoprazole (4) and rabeprazole (5). On the other hand, the reversible class compounds include a wide variety of structures, such as imidazopyridine: SCH28080 (6, 7) and pumaprazole (8) and arylquinoline: SK&F96067 (9). The covalent classes of compounds are used clinically, but chronic treatment is restricted due to development of carcinoid-like changes in the gastric mucosa of many species (10, 11). It is considered that the development of carcinoid is based on continued hypergastrinemia, resulting from long lasting acid inhibition (12). For short term acid inhibition, the reversible type of proton pump inhibitors is expected to overcome the problem of benzimidazole derivatives. Based on this concept, several companies are attempting to develop reversible class compounds. However, none have been approved for clinical use because unexpected side effects such as liver toxicity

were found during clinical trials in some cases.

We also tried to develop a new generation of proton pump inhibitors, and we found that 3-amino-5-methyl-2(2-methyl-3-thienyl)-imidazo[1,2-*a*]thieno[3,2-*c*]pyridine, SPI-447, strongly inhibited gastric H^+,K^+ -ATPase (13). However, the detailed mechanism of its inhibition has not been determined.

Therefore, in the present study, we tried to clarify the mechanism of inhibition of SPI-447 on gastric H^+,K^+ -ATPase.

MATERIALS AND METHODS

Materials

SPI-447 and SCH28080 (2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile) were synthesized by our company, and omeprazole was purchased from Union Quimico Farmaceutoca, S.A. (Barcelona, Spain). The chemical structures of test compounds are presented in Fig. 1. The purities of SPI-447 and SCH28080 determined by high-performance liquid chromatography were over

Fig. 1. The chemical structures of SPI-447, omeprazole and SCH28080.

99.9%. These agents were dissolved in dimethylsulfoxide (DMSO; Wako Pure Chemical Co., Osaka). DMSO was added to the reaction medium at a final concentration of 1%, which had no effect on ATPase activity. Na^+, K^+ -ATPase from porcine cerebral cortex was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

Preparation of gastric vesicles

Gastric vesicles were prepared from porcine fundic mucosa according to the method of Hongo et al. (14). Fresh porcine stomachs were obtained from the slaughterhouse (Matsubara Meat Plant, Osaka). The fundic mucosa was removed from the underlying muscular layer, minced with scissors, and homogenized in 0.25 M sucrose, 2 mM MgCl_2 , 1 mM EGTA and 2 mM Hepes-Tris at pH 7.4. The homogenate was centrifuged at $9,000 \times g$ for 15 min, and the supernatant obtained was then centrifuged at $20,000 \times g$ for 15 min. The resulting supernatant was centrifuged at $78,000 \times g$ for 1 h to obtain the crude microsomal pellet. For Ficoll gradient centrifugation, the crude microsomes (11 mg/ml) were layered on a single step gradient composed of 7% (w/v) Ficoll in sucrose buffer and centrifuged at $105,000 \times g$ for 1 h. The membrane fraction at the sucrose-Ficoll interface was collected. When intact vesicles were required for acidification studies, this fraction was stored at -80°C until use. To increase the permeability of the vesicle to K^+ , this fraction was diluted tenfold with sucrose buffer and then centrifuged at $125,000 \times g$ for 90 min. The resulting lyophilized vesicles were resuspended in 0.25 M sucrose and stored at -80°C until use. All procedures were performed at $0-4^\circ\text{C}$. The protein content of the H^+, K^+ -ATPase preparations was determined by the

method of Lowry et al. (15) using bovine serum albumin as a standard.

Determination of gastric H^+, K^+ -ATPase activity

H^+, K^+ -ATPase activity was determined by quantifying enzymatic inorganic phosphate release from ATP. Briefly, lyophilized vesicles ($13 \mu\text{g}$ protein) were preincubated for 30 min at 37°C in 50 mM Hepes-Tris (pH 7.4), 5 mM KCl and 2 mM MgCl_2 in the presence of test compound or vehicle. The reaction was initiated by the addition of 2 mM ATP (final concentration indicated). The volume of each component is as follows: buffering solution: $800 \mu\text{l}$, enzyme: $90 \mu\text{l}$, test compound or vehicle: $10 \mu\text{l}$, ATP: $100 \mu\text{l}$. It was run at 37°C for 15 min and stopped by the addition of 10% chilled trichloroacetic acid. The amount of released inorganic phosphate was measured by the method of Fiske and Subbarow (16). K^+ -stimulated activity was estimated as the difference between the activity in the presence of 2 mM MgCl_2 plus 5 mM KCl and basal activity (Mg^{2+} -ATPase) in the presence of 2 mM MgCl_2 alone. In our system, K^+ -stimulated and basal (Mg^{2+}) ATPase activities were 31.3 ± 3.0 and $37.0 \pm 0.3 \mu\text{M}$ Pi release/mg protein per hour, respectively. None of the compounds, at the highest concentrations tested, had any significant effect on the basal ATPase activity.

To study the effect of SH-compounds on the anti- H^+, K^+ -ATPase activities of test compounds, enzyme activities were determined in the presence of $100 \mu\text{M}$ glutathione, which had no effect on gastric H^+, K^+ -ATPase activity. In this case, 50–80% inhibitory concentrations of SPI-447, SCH28080 and omeprazole were chosen in our system; i.e., SPI-447 and SCH28080 were $20 \mu\text{M}$ and omeprazole was $60 \mu\text{M}$. The other conditions were described above.

The influence of SCH28080 on anti- H^+, K^+ -ATPase action of SPI-447 was examined according to the method of Yonetani (17). Effects of SPI-447 ($0-8 \mu\text{M}$) on H^+, K^+ -ATPase activity were determined in the presence of different concentrations of SCH28080 ($0-0.8 \mu\text{M}$). The other condition was mentioned above.

In another series of experiments, we examined the dilution effect of the test compounds on anti- H^+, K^+ -ATPase activity (18). Briefly, lyophilized gastric vesicles ($100 \mu\text{g}$ protein/ml) were preincubated with SPI-447, SCH-28080 or omeprazole at 37°C for 30 min in 50 mM Hepes-Tris buffer (pH 7.4), 2 mM MgCl_2 and 5 mM KCl. Aliquots containing $50 \mu\text{g}$ (undiluted group) and $2 \mu\text{g}$ (diluted group) protein were taken and assayed for H^+, K^+ -ATPase activity. Thus, 25-fold dilution was performed.

Determination of H^+, K^+ -ATPase-mediated H^+ -transport

The hydrogen ion transport in intact gastric vesicles was monitored from the distribution and quenching of the fluorescent probe acridine orange, which being a weak

base, accumulates in acidic compartments where its fluorescent signal is quenched (19). Assays were performed in quartz fluorimeter cuvettes at 20°C in a total volume of 1 ml. Assay reactions contained 10 mM Pipes-Tris (pH 7.0), 150 mM KCl, 2 mM MgSO₄, 2 mM ATP, 9 μM valinomycin, intact gastric vesicles (50 μg protein/assay) and 4 μM acridine orange (final concentrations indicated). The volume of each component is as follows: buffering solution: 800 μl, enzyme: 90 μl, test compound or vehicle: 10 μl, ATP: 100 μl. Fluorescence was monitored by a P-777 fluorimeter (Jasco, Tokyo) equipped with a recorder. Wave length and slit width were as follows: for excitation, 490 nm and 5 nm; for emission, 540 nm and 2.5 nm. Reactions were initiated by addition of 2 mM ATP to cuvettes containing all the other components. When the acridine orange uptake rate stabilized, test compounds were added to the reaction medium 10 min before ATP addition. Generally, the reaction was performed at pH 7.4. Only in the case of omeprazole was the reaction performed at pH 6.8, due to its weak effectiveness in this system.

In another series of experiments, we determined the effect of K⁺ on the inhibition of H⁺,K⁺-ATPase-mediated H⁺-transport by test compounds. Intact vesicles (50 μg protein) were preincubated in reaction medium containing SPI-447 (1 μM), omeprazole (10 μM), or SCH28080 (0.3 μM) in the presence of 15 mM KCl. The reaction was initiated by the addition of 2 mM ATP, and fluorescent quenching of acridine orange was monitored. Ten minutes after ATP addition, a high concentration KCl solution was added to increase the total K⁺-concentration (final concentration of 150 mM). In the case of omeprazole, KCl solution was added 30 min after ATP addition because the inhibitory effect of omeprazole was gradual.

Determination of Na⁺,K⁺-ATPase activity

For estimating the specificity of test compounds for gastric H⁺,K⁺-ATPase, the effects on Na⁺,K⁺-ATPase activity were determined. Na⁺,K⁺-ATPase was derived from porcine cerebral cortex (Sigma). Na⁺,K⁺-ATPase activity was determined by inorganic phosphate release from ATP as an ouabain-sensitive ATPase activity. Na⁺,K⁺-ATPase (13 μg protein) was preincubated with SPI-447, omeprazole or SCH28080 with or without 100 μM ouabain for 30 min in reaction buffer containing 50 mM Tris-Hepes (pH 7.4), 150 mM NaCl, 5 mM KCl and 2 mM MgCl₂. The reaction was started by adding 2 mM ATP and stopped by adding 10% TCA 30 min later (final concentrations indicated). The volume of each component is as follows: buffering solution: 800 μl, enzyme: 90 μl, test compound or vehicle: 10 μl, ATP: 100 μl. In our system, ouabain-sensitive Na⁺,K⁺-ATPase activity was 40.1 ± 1.7 μM Pi release/mg protein per hour.

Statistical analyses

All data are presented as the mean ± S.E.M. of three different experiments. The concentrations that inhibits ATPase activity by 50% (IC₅₀) and 95% confidence limit were calculated by the Litchfield-Wilcoxon method. Statistical analysis was performed by Student's *t*-test, values of *P* < 0.05 being regarded as significant.

RESULTS

Effects of SPI-447, omeprazole and SCH28080 on H⁺,K⁺-ATPase activity

K⁺-stimulated ATPase activity was 30.6 ± 4.3 μM Pi release/mg protein per hour. SPI-447, omeprazole and SCH28080 inhibited gastric H⁺,K⁺-ATPase activities in a concentration dependent manner (Fig. 2). The inhibitory intensity of SPI-447 was similar to that of SCH28080, but stronger than that of omeprazole. The IC₅₀ values of SPI-447, omeprazole and SCH28080 were 5.2 (0.87–31.2), 35.0 (5.0–81.6) and 5.2 (1.16–23.4) μM, respectively.

The ATPase activity was not changed, when the pH of the reaction medium was decreased at 6.8, the activity being 30.2 ± 2.4 μM Pi release/mg protein per hour. The inhibitory activities of these compounds became strong in the pH 6.8 medium. The IC₅₀ values of SPI-447, omeprazole and SCH28080 were 1.1 (0.31–3.85), 8.2 (2.34–28.7) and 0.68 (0.14–3.4) μM, respectively (Table 1).

Effects of SPI-447, omeprazole and SCH28080 on H⁺,K⁺-ATPase mediated H⁺ transport

When ATP was added to the reaction medium, the

Fig. 2. Effects of SPI-447 (○), omeprazole (●) and SCH28080 (▲) on the activity of H⁺,K⁺-ATPase isolated from porcine gastric mucosa. Lyophilized gastric vesicles (13 μg protein) were preincubated with the indicated concentrations of test compounds, and then the remaining ATPase activity was determined. Other conditions are described in Materials and Methods. Data are presented as the mean ± S.E.M. of 3 experiments.

Table 1. Effects of SPI-447, omeprazole and SCH28080 on the activity of gastric H^+,K^+ -ATPase

Compound	pH 7.4	IC ₅₀ (μ M)	pH 6.8
SPI-447	5.2 (0.87–31.2)		1.1 (0.31–3.85)
Omeprazole	35.0 (15.0–81.6)		8.2 (2.34–28.7)
SCH28080	5.2 (1.16–23.4)		0.68 (0.14–3.4)

Anti- H^+,K^+ -ATPase activities of test compounds were determined both at pH 7.4 and 6.8. Data are presented at the IC₅₀ value and 95% confidence limits.

fluorescent intensity was promptly decreased, indicating H^+,K^+ -ATPase mediated H^+ -transport (Fig. 3). Pretreatment with SPI-447 inhibited this transport in a concentration-dependent manner. A similar phenomenon was observed in the case of SCH28080 pretreatment. In contrast, the inhibitory action of omeprazole was observed after H^+ -transport occurred.

Effect of KCl on the inhibition of H^+,K^+ -ATPase-mediated H^+ transport by SPI-447, omeprazole and SCH28080

Pretreatment with SPI-447 or SCH28080 inhibited the H^+,K^+ -ATPase-mediated H^+ -transport. When a high concentration KCl solution was added after ATP addition, the inhibitory effects of SPI-447 and SCH28080 were sup-

pressed (Fig. 4). In the case of omeprazole pretreatment, the addition of high concentration KCl had no effect on its inhibitory action.

Effect of SH-compounds on anti- H^+,K^+ -ATPase actions of SPI-447, omeprazole and SCH28080

The enzyme activity of the control group was 26.4 ± 5.7 μ M Pi release/mg protein per hour. Glutathione (100 μ M) had no effect on this enzyme activity (25.6 ± 4.5 μ M Pi release/mg protein per hour, Fig. 5). The inhibitory actions of SPI-447 and SCH28080 on enzyme activities were not influenced by glutathione treatment, whereas the anti- H^+,K^+ -ATPase action of omeprazole was significantly suppressed.

Effect of dilution on the anti- H^+,K^+ -ATPase action of SPI-447, omeprazole and SCH28080

The enzyme activity of undiluted group (50 μ g group) was 26.9 ± 1.8 μ M Pi release/mg protein per hour. SPI-447, omeprazole and SCH28080 inhibited this enzyme activity, the inhibitory rates being $70.2 \pm 4.5\%$, $50.0 \pm 11.5\%$ and $88.1 \pm 2.7\%$, respectively (Fig. 6). After 25-fold dilution, the enzyme activity was not changed (25.1 ± 0.9 μ M Pi release/mg protein per hour), compared with the undiluted group. The inhibitory actions of SPI-447 and SCH28080 were significantly attenuated by dilution, the

Fig. 3. Effects of SPI-447 (A), omeprazole (B) and SCH28080 (C) on the H^+,K^+ -ATPase mediated H^+ transport. Intact vesicles (50 μ g protein) were preincubated in 1 ml reaction medium containing various concentrations of test compounds. The reaction was initiated by addition of 2 mM ATP, and fluorescent quenching of acridine orange was monitored. Other conditions are described in Materials and Methods. Data are the typical pattern observed in three experiments and show the fluorescent intensity of every 2 min. The concentrations of test compounds were as follows: ●: control and ○: 0.3, ▲: 1, △: 3, ■: 10, □ 30 μ M.

Fig. 4. Effect of KCl on the inhibition of H^+,K^+ -ATPase mediated H^+ transport by SPI-447, omeprazole or SCH28080. Intact vesicles ($50 \mu\text{g}$ protein) were preincubated in reaction medium containing SPI-447 ($1 \mu\text{M}$, A), omeprazole ($10 \mu\text{M}$, B) or SCH28080 ($0.3 \mu\text{M}$, C) in the presence of 15 mM KCl. The reaction was initiated by addition of 2 mM ATP, and fluorescent quenching of acridine orange was monitored. Ten minutes after ATP addition, KCl solution (final concentration: 135 mM) was added. In the case of omeprazole, KCl solution was added 30 min after ATP addition. Other conditions are described in Materials and Methods. Data are the typical pattern observed in three experiments and show the fluorescent intensity of every 2 min . $\bullet$: Control, $\circ$: no KCl addition, $\blacktriangle$: KCl addition.

Fig. 5. Effect of glutathione (GSH) on the inhibition of H^+,K^+ -ATPase activity by SPI-447, omeprazole and SCH28080. Lyophilized vesicles ($13 \mu\text{g}$ protein) were preincubated with SPI-447 ($20 \mu\text{M}$), omeprazole ($60 \mu\text{M}$), or SCH28080 ($20 \mu\text{M}$), in the absence or presence of $100 \mu\text{M}$ glutathione, and then the remaining ATPase activity was determined. Other conditions are described in Materials and Methods. Data are presented as the mean $\pm$ S.E.M. of 3 experiments. *Significantly different from GSH-untreated group, at $P < 0.05$.

Fig. 6. Effect of dilution on the inhibition of H^+,K^+ -ATPase activity by SPI-447, omeprazole and SCH28080. Lyophilized vesicles ($100 \mu\text{g}$ protein) were preincubated at 37°C for 30 min with SPI-447 ($20 \mu\text{M}$), omeprazole ($60 \mu\text{M}$) or SCH28080 ($20 \mu\text{M}$). Aliquots containing $50 \mu\text{g}$ and $2 \mu\text{g}$ protein were assayed for H^+,K^+ -ATPase activity. Data are presented as the mean $\pm$ S.E.M. of 3 experiments. *Significantly different from undiluted group, at $P < 0.05$.

Fig. 7. Yonetani and Theorell analysis of the interaction between SPI-447 and SCH28080. The ATPase activity in lyophilized gastric vesicles was measured in the presence of 0–0.8 μM of SCH28080 and 1–8 μM of SPI-447. Other conditions are described in Materials and Methods. The concentrations of SPI-447 were as follows: $\bullet$: 0, $\circ$: 1, $\blacktriangle$: 2, $\triangle$: 4, $\blacksquare$: 6, $\square$: 8 μM .

inhibitory rates being 6.5–5.4% and 16.7–2.1%, respectively. However, omeprazole strongly inhibited gastric H^+K^+ -ATPase activity even after dilution, the inhibitory rate being $67.3 \pm 14.4\%$.

Interaction of SPI-447 and SCH28080 on anti- H^+K^+ -ATPase action

The effect of SPI-447 on enzyme activity was determined in the presence of different concentrations of SCH28080, and Yonetani-Theorell analysis was performed. Each line ob-

tained from different concentrations of SPI-447 was parallel, showing that the anti- H^+K^+ -ATPase actions of SPI-447 and SCH28080 were additive (Fig. 7).

Effects of SPI-447, omeprazole and SCH28080 on Na^+K^+ -ATPase activity

SPI-447 and SCH28080 had no effect on Na^+K^+ -ATPase activity at the concentration of 100 μM , indicating that these compounds had specificity on gastric H^+K^+ -ATPase. In contrast, omeprazole inhibited it in a concentration-dependent manner (Fig. 8). The specificity was only 2.5-fold, the IC_{50} value being 68.0 (22.7–204) μM for Na^+K^+ -ATPase and 27.5 (9.2–82.5) μM for H^+K^+ -ATPase.

DISCUSSION

In this study, we tried to clarify the mechanism by which SPI-447 inhibits gastric H^+K^+ -ATPase. For this purpose, we examined the effect of SPI-447 on gastric H^+K^+ -ATPase, and compared it with those of omeprazole and SCH28080.

First, we examined the effect of these compounds on the activity of gastric H^+K^+ -ATPase and confirmed the data of Keeling et al. (7, 20) and us (13). From their IC_{50} values, the concentrations of 20 μM (SPI-447 and SCH28080) and 60 μM (omeprazole) were chosen as the 50–80% inhibitory concentrations for the other experiments.

It is well known that omeprazole is converted to its active form in acidic environments so that it demonstrates specificity to the stomach (21). A similar property was also reported for SCH28080 (22). We confirmed this property of omeprazole and SCH28080 in this study. Furthermore, the present study showed that the anti- H^+K^+ -ATPase action of SPI-447 was enhanced under acidic conditions, suggesting

Fig. 8. Effects of SPI-447 (A), omeprazole (B) and SCH28080 (C) on the activity of Na^+K^+ -ATPase from porcine cerebral cortex. Lyophilized vesicles (13 μg protein) were preincubated with the indicated concentrations of drugs in the absence or presence of 100 μM ouabain, and then the remaining ATPase activity was determined. Other conditions are described in Materials and Methods. Data are presented as the mean $\pm$ S.E.M. of 3 experiments. $\bullet$: H^+K^+ -ATPase, $\circ$: Na^+K^+ -ATPase.

that it demonstrates specificity in the stomach. Since SPI-447 is a weak base ($pK_a=5.9$), it is possible for it to be present in its protonated form under acidic conditions. Omeprazole is converted its sulfenamide form in the acidic condition (20), but SPI-447 could not be converted to the sulfenamide form because it has no sulfoxide group in its structure. Although we could not detect the protonated form, it is suggested that SPI-447 is most easily protonated at the nitrogen atom at position 1 of the imidazothienopyridine from molecular orbital analysis (AM1, MINDO). Furthermore, it was reported that protonation of the nitrogen atom at the 1-position in the imidazopyridine reinforced the inhibitory action of SCH28080 (7). Thus, SPI-447 may be an N-protonated form at position 1 in acidic conditions, and would be a stronger inhibitor, like SCH28080.

In intact gastric vesicles, the degree of acidification can be monitored directly by following the accumulation of a weak base, such as aminopyrine or the fluorescent dye acridine orange, into the vesicles. Using this system, effects of SPI-447, omeprazole and SCH28080 on acridine orange uptake were examined. All compounds inhibited the quenching of fluorescent intensity, but the inhibitory manner of each compound was quite different. This phenomenon strongly indicated that different mechanisms of pump inhibition between omeprazole and the other two compounds existed. Because the inside of the intact vesicle becomes acidic when H^+,K^+ -ATPase-mediated H^+ -transport occurs, it is possible that protonation was required for the inhibition of the proton pump by omeprazole, but not required for SPI-447 and SCH-28080. The inhibition of acridine orange uptake by SCH28080 and SPI-447 was attenuated by addition of KCl, indicating that the anti-gastric H^+,K^+ -ATPase activity of SCH28080 and SPI-447 was K^+ dependent. On the other hand, the inhibitory action of omeprazole was not attenuated by KCl addition, indicating that omeprazole inhibited gastric H^+,K^+ -ATPase in a K^+ -independent manner.

Dilution is an extremely useful method to check the reversibility of enzyme inhibition. It has already been reported that the inhibitory action of SCH28080 on gastric H^+,K^+ -ATPase was suppressed by dilution, although that of omeprazole is not influenced by dilution, as confirmed in this study (18, 21). These findings indicated that the former was a reversible inhibitor, and the latter was an irreversible one. The inhibitory action of SPI-447 was also suppressed when the reaction medium was diluted. Therefore, SPI-447 was suggested to be a reversible inhibitor of gastric H^+,K^+ -ATPase.

In this study, we confirmed that glutathione pretreatment prevented the inhibition of gastric H^+,K^+ -ATPase by omeprazole (21, 23, 24). Furthermore, we showed that SPI-447 and SCH28080 inhibited gastric H^+,K^+ -ATPase even in the presence of glutathione. These findings indicated that the inhibitory actions of SPI-447 and SCH28080 on gas-

tric H^+,K^+ -ATPase were SH-group independent. Omeprazole is known to bind cysteine at positions 813 and 892 in the primary sequence of the enzyme (25), while it was suggested that SCH28080 binds to an aspartate at position 124 and a phenylalanine at position 137 from molecular modeling studies (26). Considering that the inhibitory action of SPI-447 on gastric H^+,K^+ -ATPase was similar to that of SCH28080, but quite different from that of omeprazole, it is possible to consider that the binding site of SPI-447 to the gastric H^+,K^+ -ATPase was the same as that of SCH28080. In fact, Yonetani-Theorell analysis showed that the inhibitory actions of these compounds were additive. Although it was not confirmed, the present findings suggest that SPI-447 binds gastric H^+,K^+ -ATPase at the same site or near to the same site. Further investigations are required at this point.

It is well-known that about 60% of the amino acid residues of the H^+,K^+ -ATPase α -subunit are identical to those of the Na^+,K^+ -ATPase (27). Therefore, the effect on Na^+,K^+ -ATPase is often investigated when proton pump inhibitors are developed (18, 22). Omeprazole inhibited the activity of Na^+,K^+ -ATPase under physiological K^+ conditions, the IC_{50} ratio between H^+,K^+ -ATPase and Na^+,K^+ -ATPase being only 2.5-fold. Thus, the binding of omeprazole to gastric H^+,K^+ -ATPase was not specific. In other words, the specificity of omeprazole for gastric H^+,K^+ -ATPase is likely based on its property of requiring protonation. On the other hand, SPI-447 and SCH28080 did not inhibit the activity of Na^+,K^+ -ATPase even at a concentration of 100 μ M, indicating that the binding of SPI-447 and SCH28080 to gastric H^+,K^+ -ATPase was more specific. Considering these points, it was suggested that K^+ -competitive and reversible proton pump inhibitors are safer than irreversible inhibitors such as omeprazole.

Thus, the inhibitory mechanism of SPI-447 on gastric H^+,K^+ -ATPase was quite similar to that of SCH28080. As mentioned above, carcinoid development was reported in irreversible type proton pump inhibitors, like omeprazole (10, 11), based on hypergastrinemia from its long lasting acid inhibition (12). Therefore, chronic treatment with irreversible type of inhibitors is restricted. Yuki et al. (28) reported that the duration of acid inhibition by YM020, a reversible type of proton pump inhibitor, was shorter than that of omeprazole and longer than that of cimetidine. Furthermore, they suggested that YM020 may induce the release of less gastrin and the development of a carcinoid other than omeprazole. Therefore, it is possible that SPI-447, which also has a similar effect on gastric acid secretion in animals, may overcome the problems of the irreversible type of inhibitors.

Although the development of several K^+ -competitive, reversible proton pump inhibitors, such as SCH28080, pumaprazole and SK&F96067, have been attempted to date, there have been no successful compounds. If SPI-447 could overcome these problems, which were observed for

SCH28080, pumaprazole and SK&F96067, it would become the first reversible-type proton pump inhibitor.

In conclusion, SPI-447 is a reversible and K^+ -dependent inhibitor of gastric H^+,K^+ -ATPase. The anti-gastric H^+,K^+ -ATPase action of SPI-447 was similar to that of SCH28080, but quite different from that of omeprazole.

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REFERENCES

- Wallmark B, Jaresten BM, Larsson H, Ryberg B, Brandström A and Fellenius E: Differentiation among inhibitory actions of omeprazole, cimetidine, and SCN^- on gastric acid secretion. *Am J Physiol* **245**, G64–G71 (1983)
- Larsson H, Carlsson E, Junggren U, Olbe L, Sjöstrand SE, Skanberg I and Sundell G: Inhibition of gastric acid secretion by omeprazole in the dog and rat. *Gastroenterology* **85**, 900–907 (1983)
- Nagaya H, Satoh H, Kubo K and Maki Y: Possible mechanism for inhibition of gastric (H^+,K^+)adenosine triphosphatase by the proton pump inhibitor AG-1749. *J Pharmacol Exp Ther* **248**, 799–805 (1989)
- Kromer W, Postius S, Riedel R, Simon WA, Hannauer G, Brand U, Gonne S and Parsons ME: Bay 1023/SK&F96022INN pantoprazole, a novel gastric proton pump inhibitor, potently inhibits acid secretion but lacks relevant cytochrome P450 interactions. *J Pharmacol Exp Ther* **254**, 129–135 (1990)
- Fujisaki H, Shibata H, Oketani K, Murakami M, Fujimoto M, Wakabayashi T, Yamatsu I and Takeguchi N: Effect of the proton pump inhibitor, E3810, on gastric acid secretion and gastric and duodenal ulcers or erosions in rats. *Drug Invest* **3**, 328–332 (1991)
- Wallmark B, Briving C, Fryklund J, Munson KB, Jackson R, Mendlein J, Rabon E and Sachs G: Inhibition of gastric H^+,K^+ -ATPase and acid secretion by SCH28080, a substituted pyridyl (1,2a)imidazole. *J Biol Chem* **262**, 2077–2084 (1987)
- Keeling DJ, Laing SM and Senn-Blinger J: SCH28080 is a luminally acting, K^+ -site inhibitor of the gastric H^+,K^+ -ATPase. *Biochem Pharmacol* **37**, 2231–2236 (1988)
- Martinel J, Blum AL, Stolte M, Hartmann M, Verdu EF, Luhmann R, Dorta G and Wiesel P: Effect of pumaprazole (BY841), a novel reversible proton pump antagonist, and of omeprazole, on intragastric acidity before and after cure of *Helicobacter pylori* infection. *Alim Pharm Ther* **13**, 27–34 (1999)
- Keeling DJ, Malcolm RC, Kaing SM, Leach CA and Ife RJ: SK&F96067 is a reversible luminally acting inhibitor of the gastric H^+,K^+ -ATPase. *Biochem Pharmacol* **42**, 123–130 (1991)
- Havu N: Enterochromaffin-like cell carcinoids of gastric mucosa in rats after life-long inhibition of gastric secretion. *Digestion* **35**, Suppl 1, 42–55 (1986)
- Ekman L, Hansson E, Havu N, Carlsson E and Lundberg C: Toxicological studies on omeprazole. *Scand J Gastroenterol* **108**, Suppl 53–69 (1985)
- Creutzfeldt W: The achlorhydria-carcinoid sequence: Role of gastrin. *Digestion* **39**, 61–79 (1988)
- Ushiro T, Tsukumi Y and Tanaka H: Inhibition of gastric H^+,K^+ -ATPase by 3-amino-5-methyl-2-(2-methyl-3-thienyl)imidazo[1,2-a]thieno[3,2-c]pyridine, SPI-447. *Jpn J Pharmacol* **75**, 303–306 (1997)
- Hongo T, Nojima S and Setaka M: Purification and characterization of ($H^+ + K^+$)-ATPase from hog gastric mucosa. *Jpn J Pharmacol* **52**, 295–305 (1990)
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275 (1951)
- Fiske CH and Subbarow Y: The colorimetric determination of phosphorus. *J Biol Chem* **66**, 375–400 (1925)
- Yonetani T: The Yonetani-Theorell graphical method for examining overlapping studies of enzyme active centers. *Methods Enzymol* **87**, 500–509 (1982)
- Scott CK, Sundell E and Castrovillo L: Studies on the mechanism of action of the gastric microsomal ($H^+ + K^+$)-ATPase inhibitors SCH32651 and SCH28080. *Biochem Pharmacol* **36**, 97–104 (1987)
- Lee H and Forte J: A study of H^+ transport in gastric microsomal vesicles using fluorescent probes. *Biochim Biophys Acta* **508**, 339–356 (1978)
- Keeling DJ, Fallowfield C, Milliner K, Tingley SK, Ife RJ and Underwood AH: Studies on the mechanism of action of omeprazole. *Biochem Pharmacol* **34**, 2967–2973 (1985)
- Lindberg UP, Nordberg UP, Alminger T, Brandström A and Wallmark B: The mechanism of action of the gastric acid secretion inhibitor, omeprazole. *J Med Chem* **29**, 1327–1329 (1986)
- Briving C, Andersson BM, Nordberg P and Wallmark B: Inhibition of gastric H^+/K^+ -ATPase by substituted imidazo[1,2-a]pyridines. *Biochim Biophys Acta* **946**, 185–192 (1988)
- Sewing K-Fr, Beil W, Hackbarth I and Hannemann H: Effect of substituted benzimidazoles of H^+,K^+ -ATPase of isolated guinea-pig parietal cells. *Scand J Gastroenterol* **21**, Suppl 118, 52–53 (1986)
- Im WB, Blakeman DP and Sachs G: Reversal of antisecretory activity of omeprazole by sulfhydryl compounds in isolated rabbit gastric glands. *Biochim Biophys Acta* **845**, 54–59 (1985)
- Besancon ML, Shin JM, Mercier F, Munson KB, Miller M, Hersey SJ and Sachs G: Membrane topology and omeprazole labelling of the gastric H^+,K^+ -ATPase. *Biochemistry* **32**, 2345–2355 (1993)
- Munson KB, Balaji VN, Ramnarayan K and Sachs G: Extra-cytoplasmic region of H^+,K^+ -ATPase labeled by a K^+ -competitive photoaffinity inhibitor. *J Biol Chem* **266**, 18976–18988 (1991)
- Maeda M: Gastric proton pump (H^+/K^+ -ATPase): Structure and gene regulation through GATA DNA-binding protein(s). *J Biochem* **115**, 6–14 (1994)
- Yuki H, Kamato T, Nishida A, Ohta M, Shikama H, Yanagisawa I and Miyata K: Antisecretory and antiulcer effects of YM020, a new H^+,K^+ -ATPase inhibitor, in rats and dogs. *Jpn J Pharmacol* **67**, 59–67 (1995)