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Studies on the Mechanism of Action of the Gastric $H^+ + K^+$ ATPase Inhibitor KH 3218

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Abstract—The novel compound KH 3218 was synthesized and evaluated for its ability to inhibit the gastric $H^+ + K^+$ ATPase activity *in vitro* as well as to lessen gastric acid secretion *in vivo*. KH 3218 inhibited rabbit gastric $H^+ + K^+$ ATPase in a concentration and time dependent manner. IC_{50} value was estimated to be about 15 μM. The inhibition of the $H^+ + K^+$ ATPase by KH 3218 was blocked by sulfhydryl reducing agents, dithiothreitol or β-mercaptoethanol. The inhibition of the enzyme was not reversible by 50 fold dilution of the incubation mixtures, suggesting the irreversible nature of the inactivation. In the pylorus-ligated rat, KH 3218 reduced the total acid output as compared with the control. In addition, KH 3218 was capable of inhibiting *H. pylori* urease activity. These data suggest that KH 3218 is a potent inhibitor for $H^+ + K^+$ ATPase activity as well as for gastric acid secretion, and has a potential to be developed as a novel antiulcer agent.

Keywords □ $H^+ + K^+$ ATPase, gastric acid secretion, *H. pylori* urease, peptic ulcer, irreversible inactivation.

Gastric $H^+ + K^+$ ATPase is a proton pump located in the gastric parietal cells, and plays a role in the gastric acid secretion. Because $H^+ + K^+$ ATPase is involved in the terminal step of the gastric acid secretion (Sachs *et al.*, 1976), this enzyme has been considered as an important target for peptic ulcer therapy.

A selective gastric $H^+ + K^+$ ATPase inhibitor, omeprazole shows an effective antisecretory activity, and is used clinically for the treatment of peptic ulcers (Gustavsson *et al.*, 1983). However, due to its irreversible property of the inhibition, the inhibitory feedback effect of acid on gastrin secretion would be eliminated. This may lead to hypergastrinemia and gastric enterochromaffin-like (ECL) cell carcinoids (Sachs, 1986).

Recent reports proposed that *Helicobactor pylori (H. pylori)*, a Gram negative spiral bacterium is a pathogen for various gastrointestinal disorders such as gastritis, peptic ulcer disease and gastric cancer (Inouye *et al.*, 1989; Buck, 1990). Thus, the chemotherapy to eradicate *H. pylori* has been much focus among investigators. The mechanism by which *H. pylori* induces gastric ulcer is postulated to involve the activation of its urease and subsequent destruction of gastric mucosa by ammonia produced from urea (Nagata *et al.*, 1993). Therefore, the inhibition of *H. pylori* urease activity appears to be effective against its growth and the occurrence of gastric ulcer.

In the present study, we designed and synthesized a novel compound KH 3218 as a potential antiulcer agent. KH 3218 is structurally similar to another benzimidazole derivative, omeprazole but differs in the type of substituents. The effect of KH 3218 on gastric $H^+ + K^+$ ATPase activity *in vitro* and gastric acid secretion *in vivo* was studied. Additionally, its ability to inhibit *H. pylori* urease activity was examined. The effect of omeprazole was also investigated in some studies for comparable purposes.

Materials and Methods

Materials

Adenosine 5'-triphosphate (ATP, disodium salts), nigericin, trizma hydrochloride (Tris/HCl), trichloroacetic acid (TCA), magnesium chloride ($MgCl_2$), ethylenediaminetetraacetic acid (EDTA), ammonium chloride (NH_4Cl), dimethylsulfoxide (DMSO), (N-[2-hydroxyethyl]piperazine-N-'[2-ethane-sulfonic acid]) (HEPES), and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyethylene glycol 400 (PEG 400) was purchased from Junsei Chemical Co. (Japan).

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Butylacetate was purchased from Showa Chemical Co. (Japan).

Preparation of gastric mucosal fraction containing the $H^+ + K^+$ ATPase

The fundic regions of 6 New Zealand White Rabbits (2~3 kg) were obtained. The mucosa was scraped from the connective tissue of the stomach wall, and was homogenized in approximately 5 volume of 0.25 M sucrose containing 2 mM HEPES, 2 mM $MgCl_2$, 2 mM EDTA, 40 mM Tris/HCl, pH 7.4 using teflon-glass homogenizer (ten strokes at approximately 2000 rpm). The homogenate was centrifuged for 30 min at 10,000 ×g, and the pellets discarded.

The supernatant was further centrifuged for 60 min at 100,000×g. The pellets were resuspended in a minimum volume of 40 mM Tris/HCl buffer (pH 7.4), and stored at −70℃ until use. The protein concentration of the preparation was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

$H^+ + K^+$ ATPase assay

Enzyme preparation (25 μg) was incubated at 37℃ in 250 μl of a medium consisting of 40 mM Tris/HCl, pH 7.4, 4 mM $MgCl_2$, 5 μg/ml nigericin in methanol, 6.7 mM Na_2ATP, and with or without 48 mM KCl and 6 mM NH_4Cl. Specific $H^+ + K^+$ ATPase activity was determined after substracting the basal enzyme activity which was measured without KCl and NH_4Cl. After incubation for 30 min, the reaction was terminated by the addition of 30% cold TCA, and centrifuged. The inorganic phosphate released in the supernatant was determined by the method of Yoda and Hokin (1970). Assay medium for the $H^+ + K^+$ ATPase activity contained 2% methanol, which did not affect the enzyme activity.

Inactivation of the $H^+ + K^+$ ATPase activity by KH 3218

$H^+ + K^+$ ATPase activity was determined as described above in the presence or absence of various concentrations of KH 3218 for the indicated time of incubation. The compound was dissolved in DMSO, which, at the concentration present in the reaction mixture (2%), did not affect the enzyme activity.

Reversibility of the KH 3218-mediated inactivation of $H^+ + K^+$ ATPase

In order to determine the reversibility of the inhibition of $H^+ + K^+$ ATPase activity by KH 3218, the following method was used. $H^+ + K^+$ ATPase preparation (50 μg/ml) was preincubated with KH 3218 (0.25 mM) in 40 mM Tris/HCl buffer, pH 7.4 at 37℃. After 10 min, aliquots containing 5 μg protein were taken and assayed for the $H^+ + K^+$ ATPase activity, and remaining aliquot was diluted 50 fold in the same buffer. After 1 hr in the 50 fold dilution, aliquots were taken again for the $H^+ + K^+$ ATPase assay as described above.

Protection of the KH 3218-mediated inactivation of $H^+ + K^+$ ATPase

The ability of various agents to protect $H^+ + K^+$ ATPase against inactivation by KH 3218 was tested. $H^+ + K^+$ ATPase preparation was preincubated with the potential protecting agents at 37℃ for 10 min followed by incubation with 0.35 mM KH 3218. After 10 min, aliquots were taken and assayed for the $H^+ + K^+$ ATPase activity as described above.

***In vivo* antisecretory effect of KH 3218**

To determine *in vivo* antisecretory effect of KH 3218, Shay method (1954) was employed with some modifications. Sprague-Dawley rats (150~250 g, male) from KRICT were deprived of food but not water for 24 hr before experiment. The animals were anesthesized with diethylether, and the pylorus was ligated. KH 3218 in PEG 400 suspension (20 mg/kg) was administered intraduodenally. After 5 hr from surgery, the stomach was isolated and gastric juice was collected. After centrifugation of gastric juice at 5000 rpm for 10 min, the supernatant was analyzed for gastric acid volume, pH, and acid output by using Orion 960 autochemistry analyzer.

***H. pylori* growth conditions**

H. pylori (ATCC 43504) was cultured either on tryptic soy agar supplemented with 10% horse serum for 48 or 72 hr at 37℃ under microaerophilic condition or in Mueller Hinton broth containing 5% horse serum for 48 hr with shaking (120 rpm) at 37℃. After centrifugation for 10 min at 4℃ (10,000×g) of cultures, cells were washed and suspended with 25 mM potassium phosphate buffer (pH 6.8). The crude extract obtained from recentrifugation (20,000×g, 4℃) was used for the source of the *H. pylori* urease.

Urease assay

Urease activity was assayed by using the method of Nagata *et al.* (1993) with some modifications. 25 μl of crude urease was preincubated at 37℃ for 10 min with various amounts of KH 3218. After incubation, 50 μl of 0.1 M urea was added and reacted for 30 min. Aliquots (20 μl) were taken at every 10 min and the reaction product ammonia was quantitated with urea nitrogen (Sigma). IC_{50} was estimated by linear regression analysis.

Results

Inactivation of $H^+ + K^+$ ATPase with KH 3218

As shown in Fig. 1, KH 3218 caused a concentration

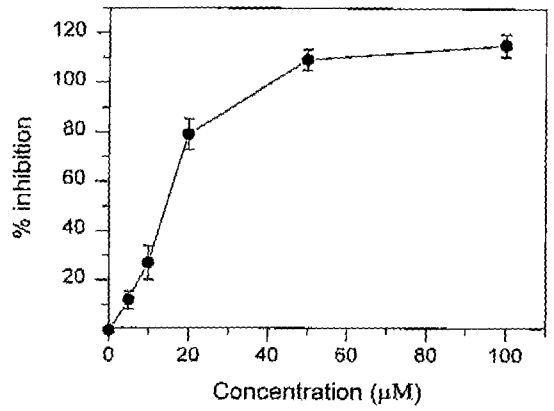

Fig. 1. Concentration dependent inactivation of rabbit gastric $H^+ + K^+$ ATPase by KH 3218. Rabbit gastric $H^+ + K^+$ ATPase was incubated at 37℃ in the presence of various concentrations of KH 3218 for 10 min. The remaining $H^+ + K^+$ ATPase activity was measured as described under Materials and Methods. Results are the mean± SD of three determinations using the same preparation of the gastric $H^+ + K^+$ ATPase.

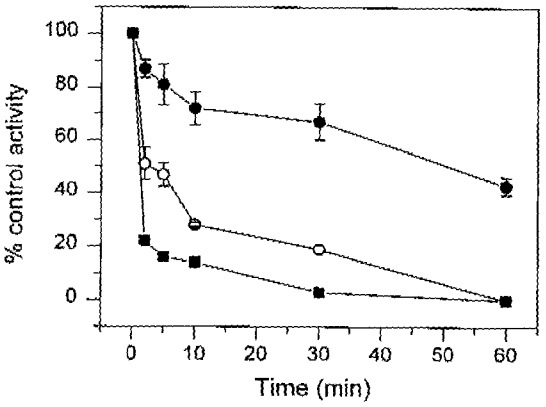

Fig. 2. Time dependent inactivation of rabbit gastric $H^+ + K^+$ ATPase by KH 3218. The reaction mixture contained rabbit gastric $H^+ + K^+$ ATPase and the indicated concentrations of KH 3218: (●) 0.2 mM; (○) 0.25 mM; (■) 0.35 mM. At the indicated intervals, aliquots were removed and the remaining enzyme activity was determined as described under Materials and Methods. Results are the mean± SD of three determinations using the same preparation of the gastric $H^+ + K^+$ ATPase. Enzyme activities are expressed as a percentage of control activity determined in the absence of KH 3218.

dependent loss of $H^+ + K^+$ ATPase activity. Total inactivation of $H^+ + K^+$ ATPase activity occurred when it was incubated with 50 μM KH 3218 for 30 min. The IC_{50} value was estimated to be about 15 μM. This value is similar to that of omeprazole, the known $H^+ + K^+$ ATPase inhibitor. In addition, preincubation of $H^+ + K^+$ ATPase with KH 3218 resulted in a time dependent inhibition of $H^+ + K^+$ ATPase activity (Fig. 2).

To investigate whether the inhibition of the $H^+ + K^+$ ATPase by KH 3218 was reversible, the $H^+ + K^+$ ATPase activity was measured before and after dilution. Preincubation with 0.25 mM KH 3218 for 10 min resulted in the inactivation of $H^+ + K^+$ ATPase activity to 28% of control activity. The enzyme activity was not restored upon 50 fold dilution of the incubation mixtures (Fig. 3), indicating that the inactivation of $H^+ + K^+$ ATPase by KH 3218 was irreversible. Furthermore, when measured 1 hr after the 50 fold dilution, the inactivated enzyme activity was not recovered while control activity remained the same (data not shown). As a further test for the irreversibility of the inactivation, gel filtration method was employed to remove the free KH 3218 from the incubation mixture treated with the compound. Consistent with the results from the dilution method, the inhibition of $H^+ + K^+$ ATPase activity was not reversed, supporting the irreversible nature of the inactivation (data not shown).

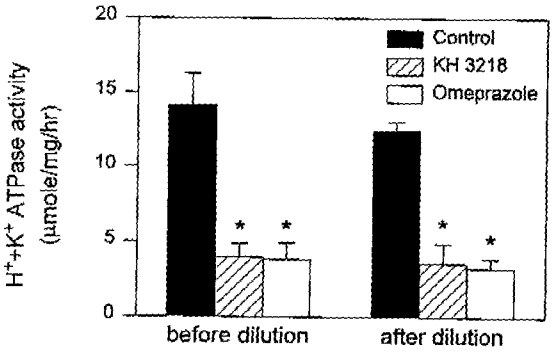

Fig. 3. Irreversibility of $H^+ + K^+$ ATPase inactivation by KH 3218. Rabbit gastric $H^+ + K^+$ ATPase preparation was incubated at 37℃ with either 0.25 mM KH 3218 or 0.25 mM omeprazole. After 10 min, aliquots were taken and the remaining aliquot was diluted 50 fold. Results are the mean± SD of three determinations. *$p<0.01$ vs Control.

Protection of KH 3218-mediated $H^+ + K^+$ ATPase inactivation by various agents

Previous reports have indicated that inhibition of $H^+ + K^+$ ATPase by omeprazole is due to the modification of essential sulfhydryl groups in the enzyme (Lorentzon *et al.*, 1985; Wallmark *et al.*, 1984; Im *et al.*, 1985). In the present study, the effects of sulfhydryl reducing agents, dithiothreitol (DTT) and β-mercaptoethanol (β-ME) on the KH 3218-mediated inhibition of $H^+ + K^+$ ATPase activity were investigated. As shown in Table I, addition of 0.5 mM DTT or 0.5 mM β-ME to the incubation mixture prior to the addition of 0.35 mM KH 3218 protected $H^+ + K^+$ ATPase from the inactivation by KH 3218. These data suggest that KH 3218 may interact with and covalently bind to the sulfhydryl groups of the $H^+ + K^+$ ATPase by a similar mechanism as for omeprazole.

Table I. Protection of $H^+ + K^+$ ATPase activity by DTT and β-ME against KH 3218-induced inhibition[a].

Conditions	% control activity
0.35 mM KH 3218	13± 2.1
0.35 mM KH 3218+0.1 mM DTT	60± 6.0[b]
0.35 mM KH 3218+0.5 mM DTT	90± 9.0[b]
0.35 mM KH 3218+0.1 mM β-ME	63± 6.7[b]
0.35 mM KH 3218+0.5 mM β-ME	98± 11[b]

[a]Rabbit gastric $H^+ + K^+$ ATPase preparation was incubated with 0.35 mM KH 3218 and the indicated protecting agents. At the end of 10 min incubation, aliquots were taken and the remaining enzyme activity was determined as described under Materials and Methods. Each value is the mean± SD of three determinations. [b]P<0.01 by Student's two tailed *t*-test.

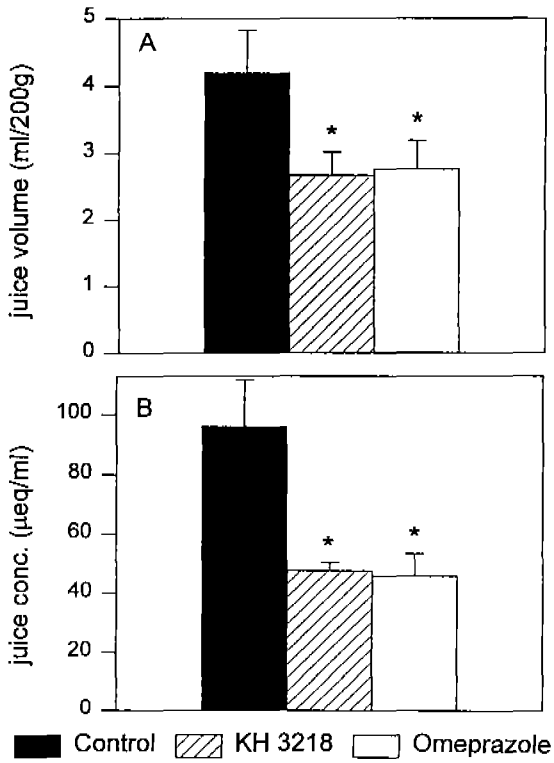

Fig. 4. Effect of *i.d.* KH 3218 on gastric acid secretion in pylorus-ligated rats. A. effect of KH 3218 on gastric acid volume. B. effect of KH 3218 on gastric acidity. KH 3218 or omeprazole (20 mg/kg, *i.d.*) was administered into the pylorus-ligated SD rats. After 5 hr from surgery, gastric content was collected. The volume and acidity of gastric acid was measured. Each value is the mean± SD of five determinations. *p<0.01 vs Control.

In vivo antisecretory effect of KH 3218

In order to determine whether KH 3218 has an antisecretory effect *in vivo*, KH 3218 (20 mg/kg, *i.d.*) was given to rats, and its effect on the gastric acid secretion was examined. Gastric acid secretion was markedly inhibited by the intraduodenal administration of KH 3218 (Fig. 4). This reduction in acid output was due to the decreases in both the volume (Fig. 4A) and the concentration of gastric juice (Fig. 4B). The observed degree of the inhibition by KH 3218 was comparable to that by omeprazole. Based on the results presented in Fig. 4, total acid output was calculated and presented in Table II. Relative acid output of KH 3218 was approximately 1.05, implying that the inhibition of gastric acid secretion *in vivo* was as potent as that by omeprazole.

Table II. Total acid output in the pylorus-ligated SD rats by either omeprazole or KH 3218 administration[a].

Conditions	Total acid output (μeq/5 hr)
control	409± 114
omeprazole-treated	123± 34.4[b]
KH 3218-treated	110± 61.9[b]

[a]Total acid output was calculated by multiplying the volume and the concentration of gastric acid obtained from Fig. 4. Each value represents the mean± SD of five determinations. [b]P<0.01 by Student's two tailed *t*-test.

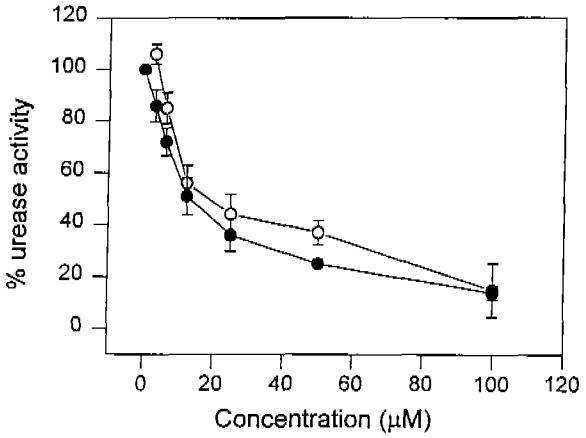

Fig. 5. Concentration dependent inhibition of *H. pylori* urease either by KH 3218 or by omeprazole (OMZ). *H. pylori* urease was preincubated with various concentrations of either KH 3218 (●) or OMZ (○) for 10 min. The remaining urease activity was determined as described under Materials and Methods. Results are expressed as the mean± SD of three determinations.

Inhibition of *H. pylori* urease activity by KH 3218

When the optimal pH for *H. pylori* urease activity was examined, the urease activity was highest at pH 8.0, although the broad pH range (5.0~9.5) was suitable for the urease activity. On the other hand, preincubation of *H. pylori* urease at 37℃ longer than 20 min reduced the enzyme activity.

As shown in Fig. 5, KH 3218 inhibited *H. pylori* urease activity in a dose dependent manner at pH 6.0. Its IC_{50} value was estimated to be 13.9 μM, which is

similar to that of omeprazole (18.7 μM), and 2.7 fold lower than that of acetohydroxamic acid, another urease inhibitor.

Discussion

This study demonstrates that a novel compound KH 3218 is a potent inhibitor of $H^+ + K^+$ ATPase and of gastric acid secretion. KH 3218 inhibits the $H^+ + K^+$ ATPase activity in preparations of rabbit gastric microsomes in a concentration and time dependent manner with a 50% inhibition value of 15 μM. The results of the reversibility experiment have shown that the inhibition of the $H^+ + K^+$ ATPase activity by KH 3218 was not reversible by dilution, indicating the irreversible nature of the inactivation. However, taking the possible side effects of irreversible inhibition of $H^+ + K^+$ ATPase into consideration as for omeprazole, the development of reversible $H^+ + K^+$ ATPase inhibitors would be advantageous. Indeed, syntheses of the reversible $H^+ + K^+$ ATPase inhibitors have been tried although clinical trial has been discontinued (Scott *et al.*, 1987).

Omeprazole-induced $H^+ + K^+$ ATPase inactivation is blocked by coincubation with β-ME, suggesting that inhibition by omeprazole may involve its irreversible disulfide linkage with the ATPase (Keeling *et al.*, 1985). The data reported herein also demonstrate that the presence of 0.5 mM DTT or β-ME completely abolished the ability of KH 3218 to inhibit the $H^+ + K^+$ ATPase activity. These results suggest that essential cysteine residue(s) of the enzyme may be modified by KH 3218. Further studies to isolate and characterize the modified enzyme would be required to clarify this hypothesis.

In vivo studies, KH 3218 was shown to be capable of inhibiting gastric acid secretion. The reduction of gastric acid volume and concentration contributed to the decrease of total gastric acid secretion by KH 3218. The pH of gastric juice was somewhat increased from 1.71 (control treated) to 2.54 (KH 3218 treated). KH 3218- induced antisecretory effect may be due to its inhibitory action on gastric $H^+ + K^+$ ATPase activity.

The possibility that KH 3218 also inactivates *H. pylori* urease activity was investigated. KH 3218 caused a concentration dependent inhibition of *H. pylori* urease activity. The ability of KH 3218 to inhibit *H. pylori* urease activity may add to the benefit in the development of the compound as an antiulcer agent. Although KH 3218 was not much more potent than omeprazole, KH 3218 could be of interest for the treatment of peptic ulcer disease on the basis of the present study. An acute toxicity test with the compound is under progress. The results from future studies would establish whether KH 3218 exhibits favorable therapeutic effect with little toxicity on the course of peptic ulcer treatment.

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