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Effects of Newly Synthesized Benzimidazole Derivatives on Gastric H^+/K^+ ATPase

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The effects of various synthetic benzimidazole derivatives on gastric H^+/K^+ ATPase activity *in vitro* were examined. The results showed that the effects of substituents on the benzimidazole ring were not significant. However, replacement of sulfoxide connecting two ring systems to sulfide resulted in a completely inactive compound *in vitro*, suggesting the essential role of sulfoxide group in the inhibition. In addition, compounds with 5 or 6-membered oxacyclic substituents attached to the pyridine ring displayed the most effective inhibitory activity. Among these derivatives, AU-47 was the most potent, and detailed mechanistic studies with the compound were carried out. AU-47 inhibited gastric H^+/K^+ ATPase in a concentration and time dependent manner with 50 % inhibition at 6 μM. The presence of sulfhydryl reducing agents or substrate analogue protected H^+/K^+ ATPase from the inactivation. The inhibition by AU-47 was potentiated by acid pretreatment of the compound, suggesting the structural conversion of AU-47 into a more active intermediate which was favored in acidic condition. Consistent with *in vitro* results, AU-47 inhibited *in vivo* gastric acid secretion. The results suggest that AU-47 is a relevant candidate for the development of new antiulcer agent.

Key words : H^+/K^+ ATPase, Gastric secretion, Antiulcer agent, Structure-activity relationship, IC_{50}, Acidification.

INTRODUCTION

Substituted benzimidazole derivatives have been shown to be potent inhibitors of gastric acid secretion (Gustavsson *et al.*, 1983), thus being important clinical candidates for peptic ulcer diseases. Their mechanism of action was proved to be the inhibition of gastric H^+/K^+ ATPase, known as a proton pump in the parietal cell (Wallmark *et al.*, 1985). Reports have indicated that omeprazole, a prototype compound of substituted benzimidazole derivatives, is activated to the reactive intermediate under acidic condition, and then binds covalently to essential sulfhydryl group(s) of the H^+/K^+ ATPase, resulting in the inhibition of gastric acid secretion (Lorentzon *et al.*, 1985; Keeling *et al.*, 1985). However, its safety has been controversial due to the occurrence of hypergastrinemia and enterochromaffin-like (ECL) cell hyperplasia (Larsson *et al.*, 1986).

Considering the large population with peptic ulcer diseases, appropriately designed drugs would improve the therapeutic effectiveness. Analysis on the structure-activity relationship of benzimidazole derivatives revealed that three parts including a substituted pyridine ring, a substituted benzimidazole ring and CH_2SO chain connecting these two rings are required for the antisecretory effect (Brandstrom *et al.*, 1986) (Fig. 1). Contrary to this result, benzimidazole derivatives with aniline moiety in place of pyridine ring also showed potent inhibitory effect on the H^+/K^+ ATPase (Adelstein *et al.*, 1988). Based on these information, we designed structural analogues of omeprazole with the aim of the development of more effective and side effect-free antiulcer agents. Using the pharmacological screening of various synthetic benzimidazole derivatives, the structure-activity relationship of the compounds in the inhibition of gastric H^+/K^+ ATPase was studied. Of the variety of substituted benzimidazole derivatives, AU-47 having 5-

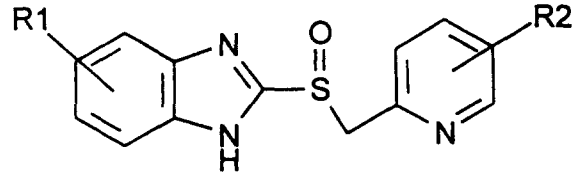

Fig. 1. Structure of substituted benzimidazole derivatives

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membered oxacyclic substituent on the pyridine ring was the most potent when tested. Now detailed pharmacological studies were performed for this compound on the gastric H^+/K^+ ATPase activity and the gastric acid secretion *in vivo*. The results suggest that AU-47 is a potent inhibitor of *in vivo* gastric acid secretion as well as *in vitro* gastric H^+/K^+ ATPase.

MATERIALS AND METHODS

Materials

Adenosine 5'-triphosphate (Na_2ATP, disodium salts), nigericin, trizma base (Tris), trichloroacetic acid (TCA), magnesium chloride ($MgCl_2$), ammonium chloride (NH_4Cl), dimethylsulfoxide (DMSO), (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethane-sulfonic acid]) (HEPES), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin, potassium chloride (KCl), sodium acetate ($C_2H_3O_2Na.3H_2O$), adenosine 5'-diphosphate (ADP, sodium salt), adenosine 5'-*O*-(3-thiotriphosphate) (ATPrS), and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dithiothreitol (DTT) and β-mercaptoethanol (β-ME) were obtained from BioRad Laboratories (Richmond, CA, USA). Polyethylene glycol 400 (PEG 400) and perchloric acid ($HClO_4$, 60%) were purchased from Junsei Chemical Co. (Japan). Butylacetate was purchased from Showa Chemical Co. (Japan). Diethylether was obtained from Oriental Chemical Industry (Korea).

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

Gastric H^+/K^+ ATPase was prepared as described previously (Forte *et al.*, 1967) with some modification (Cheon *et al.*, 1995). Briefly, the fundic mucosae of New Zealand White Rabbits (2-3 kg) were scraped and homogenized in 40 mM Tris/HCl, pH 7.4 containing 0.25 M sucrose, 2 mM HEPES, 2 mM $MgCl_2$, 2 mM EDTA. The homogenate was centrifuged for 30 min at 10,000×g, followed by subsequent centrifugation of the resulting supernatant 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°C until use. The protein concentration of the preparation was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

In vitro screening of synthetic benzimidazole derivatives by H^+/K^+ ATPase assay

In vitro inhibitory effects were determined by employing H^+/K^+ ATPase enzyme assay. The reaction mixture (200 μl) contained enzyme preparation (25 μg) in 40 mM Tris/HCl, pH 7.4, 4 mM $MgCl_2$, 5 μg/ml nigericin in methanol, and with or without 48 mM KCl and 6 mM NH_4Cl. The synthetic compounds (100 μM) were dissolved in DMSO, and preincubated with the enzyme preparation at 37°C for 30 min. Final concentration (2%) of DMSO in the reaction mixture did not affect the enzyme activity. After initiation of the reaction by adding 6.7 mM Na_2ATP (50 μl), the reaction mixture was further incubated for 30 min. The reaction was terminated by the addition of 30% cold TCA (50 μl), and centrifuged. The inorganic phosphate released from Na_2ATP in the supernatant was determined spectrophotometrically according to the Yoda and Hokin (1970). Specific H^+/K^+ ATPase activity was determined after substracting the basal enzyme activity which was measured in the absence of KCl and NH_4Cl. Inhibition was calculated as percent inhibition against maximal stimulation, and IC_{50} values were obtained from a typical dose-response curve. Assay medium contained 2% methanol, which did not affect the enzyme activity.

Detailed *in vitro* studies of AU-47, a representative compound of the benzimidazole derivatives

The ability of various agents to protect H^+/K^+ ATPase against inactivation by AU-47 was tested. H^+/K^+ ATPase preparation was preincubated with the potential protecting agents at 37°C for 5 min followed by incubation with 20 μM AU-47. After 30 min, aliquots were taken and assayed for the H^+/K^+ ATPase activity as described above. To examine the effect of acidification of AU-47 on the inactivation of H^+/K^+ ATPase activity, AU-47 (0.25 mM) was held at 37°C for 20 min in either 40 mM sodium acetate (pH 4.0) or 40 mM Tris/HCl (pH 6.3) or 40 mM Tris/HCl (pH 7.4) buffer. Each solution was then added into incubation mixture containing gastric H^+/K^+ ATPase preparation, which resulted in the one hundred fold dilution of AU-47. The assay mixture was then incubated at 37°C for 30 min, and assayed for the remaining H^+/K^+ ATPase activity.

In vivo antisecretory effect of AU-47

In vivo antisecretory effect of AU-47 was determined using pylorus-ligated rat according to Shay *et al.* (1954). Sprague-Dawley rats (150-250 g, male) were obtained from KRICT, and food was withdrawn for 24 hr before experiment. The pylorus of the rats was ligated under diethylether anesthesia. AU-47 in PEG 400 suspension (20 mg/kg) or omeprazole (20 mg/kg) was administered intraduodenally. Control groups were given PEG 400 solution alone. Five hrs after the surgery, the stomach was isolated and the accumulated gastric juice was collected. After centrifugation of gastric juice at 5000 rpm for 10 min,

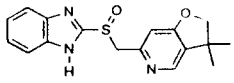
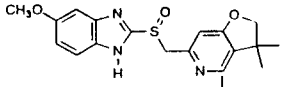
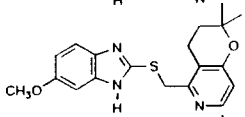
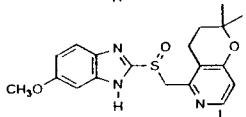
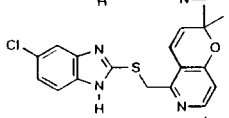
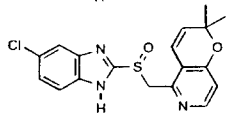
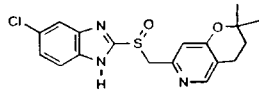
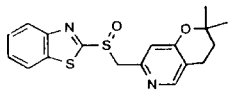
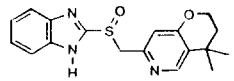
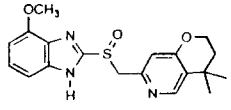
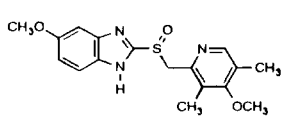
the supernatant was analyzed for gastric acid volume, pH, and acid output by using Orion 960 autochemistry analyzer. Total acid output was calculated from the product of the gastric acid concentration and volume of gastric juice.

RESULTS

Structure-activity relationship of benzimidazole derivatives in the inhibition of H^+/K^+ ATPase

The substituted benzimidazole derivatives were synthesized and their effects on gastric H^+/K^+ ATPase activity were examined (Table I). The basic structure of the substituted benzimidazole derivatives is shown in Fig. 1. The compounds with different substituents (R1, either methoxy or chloride) on the benzimidazole ring showed similar inhibitory effect with the compounds without substituents (**1** vs **2**, **9** vs **10**). In addition, as observed previously, replacing either sulfoxide connecting two ring systems to sulfide (**3** vs **4**, **5** vs **6**) or benzimidazole ring to benzothiazole ring (**7** vs **8**) abolished the activity completely, indicating the presence of sulfoxide and benzimidazole ring is essential. On the other hand, the compounds with 5 or 6-membered oxacyclic substituents on the pyridine ring (R2, **1**, **9**) showed improved inhibitory potency as compared with omeprazole.

Table I. Inhibition of gastric H^+/K^+ ATPase by substituted benzimidazole derivatives

Compound	Structure	IC_{50} (μM)[a]
1		6±2.3
2		10±1.8
3		>100
4		15±2.6
5		>100
6		15±0.5
7		10±1.9
8		>100
9		7±3.2
10		9±1.6
Omeprazole		15±0.7

[a]The IC_{50} value was estimated from the dose-response curve of the H^+/K^+ ATPase enzyme assay as described in Materials and Methods. Results were expressed as the mean±SD of triplicate experiments.

Effect of AU-47 on gastric H^+/K^+ ATPase

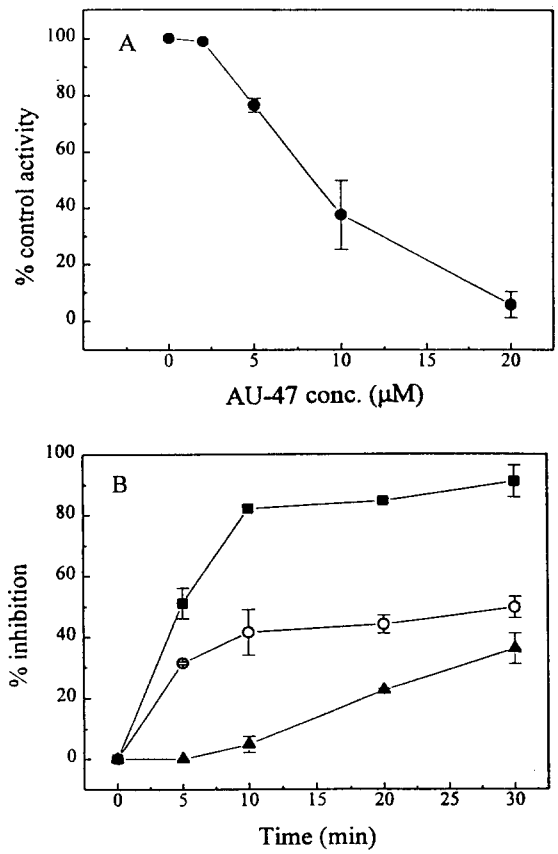


Fig. 2. A. Concentration dependent inhibition of H^+/K^+ ATPase by AU-47. H^+/K^+ ATPase preparation was incubated with various concentrations of AU-47 at 37°C. At the end of 30 min, aliquots were withdrawn and assayed for H^+/K^+ ATPase activity as described in Materials and Methods. Results are expressed as mean±SD (N=3). B. Time dependent inhibition of H^+/K^+ ATPase by AU-47. H^+/K^+ ATPase preparation was preincubated for the indicated time with AU-47; 20 μM (■), 10 μM (○), 5 μM (▲). The H^+/K^+ ATPase activity was then determined, and results are expressed as mean±SD (N=3).

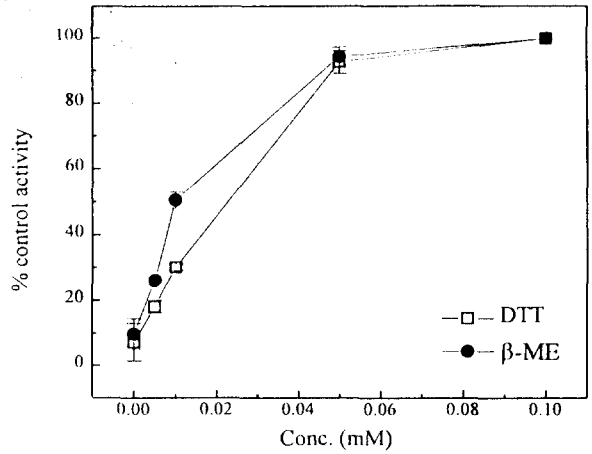


Fig. 3. Effect of DTT and β-ME on the AU-47-mediated inhibition of H^+/K^+ ATPase activity. H^+/K^+ ATPase preparation was preincubated with the indicated concentrations of either DTT (□) or β-ME (●) at 37°C for 5 min. AU-47 (20 μM) was then added into preincubation mixture and further incubated for 30 min. The H^+/K^+ ATPase activity was determined as described in Materials and Methods. Results are expressed as mean±SD (N=3).

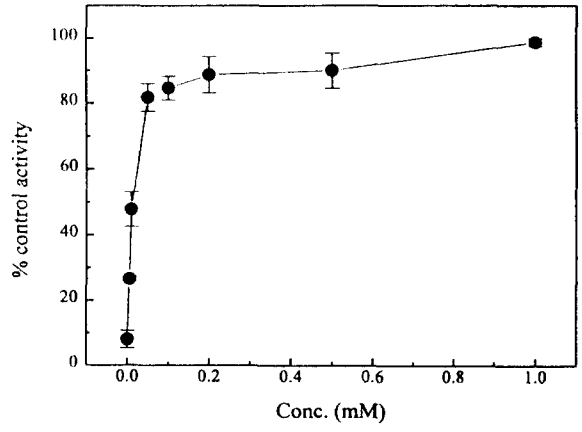


Fig. 4. Effect of ATPγS on the AU-47-mediated inhibition of H^+/K^+ ATPase activity. H^+/K^+ ATPase preparation was preincubated with various concentrations of ATPγS at 37°C for 5 min followed by incubation with 20 μM AU-47. After 30 min, aliquots were withdrawn and assayed for the H^+/K^+ ATPase activity as described in Materials and Methods. Results are expressed as mean±SD (N=3).

Among various benzimidazole derivatives screened, compound **1** was the most potent (Table I) and designated as AU-47. AU-47 contained 5-membered oxacyclic substituents on the pyridine ring and no substituent on the benzimidazole ring. Detailed studies on the AU-47-mediated inhibition of H^+/K^+ ATPase revealed that AU-47 caused a concentration and time dependent inactivation of H^+/K^+ ATPase activity with an IC_{50} value of 6 μM (Fig. 2). For comparison, omeprazole inhibited gastric H^+/K^+ ATPase activity with an IC_{50} value of 15 μM under the same experimental condition (Table I).

To investigate whether the inhibition of H^+/K^+ ATPase activity by AU-47 was mediated by the modification of cysteine residue(s) of the enzyme, the effect of sulfhydryl reducing agents on the inhibition was examined. The addition of dithiothreitol (DTT) or β-mercaptoethanol (β-ME) to the incubation mixture prior to the addition of 20 μM AU-47 protected H^+/K^+ ATPase activity in a concentration dependent manner (Fig. 3). In addition, the nonhydrolyzable substrate analogue, ATPγS protected H^+/K^+ ATPase activity from the inactivation by AU-47 (Fig. 4). On the other hand, ADP or KCl had no protective effect against AU-47-mediated inactivation of H^+/K^+ ATPase (data not shown).

Since omeprazole appeared to be more potent for the inhibition of the H^+/K^+ ATPase at lower pH values (Keeling *et al.*, 1985), the effect of the acid exposure of AU-47 on its H^+/K^+ ATPase inhibitory action was studied. Incubation of AU-47 (5 μM) at 37°C for 20 min at pH 4.0 resulted in a complete inhibition of the H^+/K^+ ATPase activity which is more potent than that obtained at higher pHs (pH 6.3 or pH 7.4) (Table II). These results suggest that some forms of acid activation occur in the inhibition process. Thus, AU-47 may be the precursor of an active inhibitor and the conversion is enhanced by acidic conditions.

Table II. Effect of transient acidification of AU-47 on its inhibitory action on gastric H^+/K^+ ATPase

pH condition	% inhibition[a]
7.4	5.3±7.9
6.3	30.7±9.1
4.0	99.8±3.4

[a]After pretreatment of AU-47 (0.25 mM) under the indicated pH conditions, each solution was added into incubation mixture and assayed for the H^+/K^+ ATPase activity. Percent inhibition was calculated against control activity measured in the absence of AU-47. Values are mean±SD of three separate experiments.

In vivo antisecretory effect of AU-47

In order to determine whether AU-47 has an antisecretory effect *in vivo*, AU-47 (20 mg/kg, *i.d.*) was given to rats, and its effect on the gastric acid secretion was examined. Total gastric acid secretion was decreased by the intraduodenal administration of AU-47 to 75% of the control (Fig. 5). The degree of acid output blockade was similar to omeprazole. The volume as well as the concentration of gastric juice was decreased, which resulted in the reduction of total acid output.

DISCUSSION

The present study was undertaken to investigate

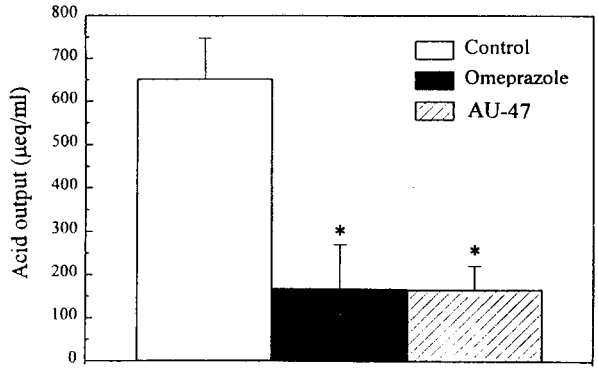


Fig. 5. Inhibition of *in vivo* gastric acid secretion by either omeprazole or AU-47. Either omeprazole (20 mg/kg) or AU-47 (20 mg/kg) in PEG 400 suspension was given to pylorus-ligated rats intraduodenally. Five hrs after administration, the gastric content was collected and analyzed for acid output. Results are expressed as mean±SD (N=5). *P<0.01 vs Control

whether the newly synthesized benzimidazole derivatives have potential to be developed as antiulcer agents. From the *in vitro* screening with the isolated gastric mucosal fraction, we analyzed structure-activity relationship of the substituted benzimidazole derivatives in the inhibition of H^+/K^+ ATPase. The present results show that the substituents on the benzimidazole ring are not important, while the introduction of 5-membered oxacyclic forms on the pyridine ring potentiated the inhibitory activity of the compound. In addition, the presence of sulfoxide chain as well as benzimidazole ring was important in the inhibitory action. Studies on the detailed structure-activity relationship including syntheses and structures will be reported (manuscript in preparation). AU-47 with 5-membered oxacyclic substituent on the pyridine ring exhibited the highest activity as a H^+/K^+ ATPase inhibitor among the compounds tested, and detailed analysis with the compound was carried out. The IC_{50} value of the AU-47 on the inhibition of H^+/K^+ ATPase was estimated to be 6 μM, which is 2.5 times more potent than omeprazole, a prototype of the H^+/K^+ ATPase inhibitors.

Similar to the published data on omeprazole (Lorentzon *et al.*, 1985), AU-47-mediated inhibition of H^+/K^+ ATPase was blocked upon preincubation with either DTT or β-ME, implying the involvement of cysteine residue(s) in the inactivation of enzyme activity. The experiments on the reversibility of the inactivation have shown that the inhibition by AU-47 was not reversible either by dilution or by gel filtration (results not shown). These results suggest that the inactivation of gastric H^+/K^+ ATPase activity by AU-47 is irreversible in nature and may involve disulfide covalent linkage with the enzyme.

The potency of AU-47 in inactivating H^+/K^+ ATPase activity was correlated with the acidity of the preincubation environment. The preincubation of AU-47 in acidic conditions (pH 4.0 or pH 6.3) resulted in a more efficient inactivation of the H^+/K^+ ATPase activity as compared with the inhibition achieved at neutral pH. This result is suggestive of the activation of AU-47 under acidic environment. Similarly, omeprazole has been reported to be converted to an acid-generated active product in the inhibition process (Keeling *et al.*, 1985). Taken together, it appears that AU-47 elicited its inhibitory activity on the gastric H^+/K^+ ATPase by the covalent modification of an acid-generated intermediate with essential cysteine residue(s) on the enzyme.

The inhibition of gastric H^+/K^+ ATPase by AU-47 was protected by low concentrations of ATPγS, a substrate analogue. This result suggests that the residue(s) modified by AU-47 reside near or at the ATP binding domain of the enzyme. If this is the case, the radiolabelled AU-47 would be a useful molecular probe for the isolation and characterization of active site peptide from H^+/K^+ ATPase. Alternatively, it remains also possible that binding of the ATPγS induces the conformational change of the H^+/K^+ ATPase, thus indirectly protecting the enzyme from the inactivation. On the other hand, the presence of ADP had no effect on the inactivation by AU-47, possibly due to the poor binding of ADP being the dephosphorylated form of the substrate.

In vivo studies using pylorus-ligated rat demonstrated that AU-47 was also a potent inhibitor of gastric acid secretion. The inhibition of gastric secretion was attributed to the reduction in the volume and concentration of gastric juice. This reduction of acid output by AU-47 probably resulted from the inhibition of gastric H^+/K^+ ATPase activity.

In summary, the present findings show that the presence of 5-membered oxacyclic form on the pyridine ring enhances the inhibitory activity to H^+/K^+ ATPase. A representative compound AU-47 was a potent and irreversible inhibitor of gastric H^+/K^+ ATPase, although the exact mechanism of the inactivation process cannot be defined with certainty. Consistent with *in vitro* data, AU-47 also inhibited gastric acid secretion *in vivo*. Based on the present results, AU-47 is worthy of continuous pharmacodynamic, pharmacokinetic and toxicity studies for assessing its clinical utility in peptic ulcer therapy.

REFERENCES CITED

Adelstein, G. W., Yen, C. H., Haack, R. A., Yu, S., Gullikson, G., Price, D. V., Anglin, C., Decktor, D. L., Tsai, H., and Keith, R. H., Substituted 2-[(2-benzimidazolylsulfinyl)methyl]anilines as potential inhibitors of H^++K^+ ATPase. *J. Med. Chem.*, 31, 1215-1220 (1988).

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram. *Biochem.*, 72, 248-254 (1976).

Brandstrom, A., Lindberg, P., Junggren, U., and Wallmark, B., Structure-activity relationships of substituted benzimidazoles. *Scand. J. Gastroenterol.*, 21 (118), 54-56 (1986).

Cheon, H. G., Kim, H. J., Yum, E. K., Cho, S. Y., Kim, D. Y., and Yang, S.I., Studies on the mechanism of action of the gastric H^++K^+ ATPase inhibitor KH 3218. *J. Applied Pharmacol.*, 3 (3), 205-209 (1995).

Forte, J. G., Forte, G. M., and Saltman, P., K^+-stimulated phosphatase of microsomes from gastric mucosa. *J. Cell Physiol.*, 69, 293-304 (1967).

Gustavsson, S., Loof, L., Adami, H. O., Nyberg, A., and Nyren, O., Rapid healing of duodenal ulcers with omeprazole. *Lancet* 2., 124-125 (1983).

Keeling, D. J., Fallowfield, C., Milliner, K. J., Tingley, S. K., Ife, R.J., and Underwood, A.H., Studies on the mechanism of action of omeprazole. *Biochem. Pharmacol.*, 34 (6), 2967-2973 (1985).

Larsson, H., Carlsson, E., Mattsson, H., Lundell, L., Sundler, F., Sundell, G., Wallmark, B., Watanabe, T., and Hakanson, R., Plasma gastrin and gastric enterochromaffin-like cell activation and proliferation. Studies with omeprazole and ranitidine in intact and antrectomized rats. *Gastroenterology*, 90, 391-399 (1986).

Lorentzon, P., Eklundh, B., Brandstrom, A., and Wallmark, B., The mechanism for inhibition of gastric H^++K^+ ATPase by omeprazole. *Biochim. Biophys. Acta*, 817, 25-32 (1985).

Shay, M., Sun, O. C. H., and Gruenstein, M., A quantitative method for measuring spontaneous gastric secretion in the rat. *Gastroenterology*, 26, 906-913 (1954).

Wallmark, B., Larsson, H., and Humble, L., The relationship between gastric acid secretion and gastric H^++K^+ ATPase activity. *J. Biol. Chem.*, 260 (25), 13681-13684 (1985).

Yoda, A., and Hokin, L. E., On the reversibility of binding of cardiotonic steroids to a partially purified (Na+K)-activated adenosinetriphosphatase from beef brain. *Biochem. Biophys. Res. Commun.*, 40, 880-886 (1970).