ELSEVIER

European Journal of Pharmacology 411 (2001) 181–186

ejp

www.elsevier.nl/locate/ejphar

Biochemical properties of a newly synthesized H^+/K^+ ATPase inhibitor, 1-(2-methyl-4-methoxyphenyl) -4-[(3-hydroxypropyl) amino]-6-methyl-2,3-dihydropyrrolo[3,2-*c*]quinoline

Hyae Gyeong Cheon [a,*], Hong Lim [b], Dong Ha Lee [b]

[a] *Pharmaceutical Screening Center, Korea Research Institute of Chemical Technology, 100 Jang-Dong, Yusong-Gu, Taejon, 305-343, South Korea*
[b] *Dongbu Hannong Chemical Co. Ltd., 103-2 Moonji-Dong, Yusong-Gu, Taejon, 305-380, South Korea*

Received 17 October 2000; received in revised form 23 November 2000; accepted 28 November 2000

Abstract

A new compound, 1-(2-methyl-4-methoxyphenyl)-4-[(3-hydroxypropyl)amino]-6-methyl-2,3-dihydropyrrolo[3,2-*c*]quinoline (DBM-819), inhibited gastric H^+/K^+ ATPase in the rabbit (EC 3.6.1.3) with an IC_{50} value of 5 μM. However, DBM-819 was a weak inhibitor of kidney Na^+/K^+ ATPase in the dog, indicating that it has selectivity for the gastric H^+/K^+ ATPase. The inhibition was reversible and non-competitive with respect to the activating cation K^+. The presence of dithiothreitol did not protect the H^+/K^+ ATPase from inactivation. The inhibition by DBM-819 was potentiated by acid pretreatment of the compound, suggesting that DBM-819 is converted into a more active intermediate under acidic conditions. The results suggest that DBM-819 is a potent, selective and reversible inhibitor of gastric H^+/K^+ ATPase, and that the essential cysteine residue may not be involved in the DBM-819-mediated inactivation of gastric H^+/K^+ ATPase.

Keywords: H^+/K^+ ATPase; Reversibility; Acid activation; Non-competitive inhibition

1. Introduction

Gastric H^+/K^+ ATPase, located in the apical membrane of parietal cells, pumps protons into the gastric lumen using energy derived from the hydrolysis of ATP, and is thus involved in gastric acid secretion (Sachs et al., 1976). Accordingly, the inhibition of gastric H^+/K^+ ATPase has been an attractive target for new peptic ulcer drugs. The genes encoding gastric H^+/K^+ ATPase have been cloned from rats (Shull, 1990; Shull and Lingrel, 1986) and humans (Maeda et al., 1990), and their amino acid sequences reveal high homology with the Na^+/K^+ ATPase. Like the Na^+/K^+ ATPase, gastric H^+/K^+ ATPase is composed of transmembrane α and β subunits, with the α subunit containing the catalytic component of the enzyme (Shin et al., 1997). The α subunit has a M.W. of 114 kDa (Shull and Lingrel, 1986) and the β subunit has a M.W. of 33 kDa (Shull, 1990).

Gastric H^+/K^+ ATPase inhibitors are the most potent suppressors of gastric acid currently known, and they have been classified into two types: one type acts irreversibly and the other type acts reversibly. Many benzimidazole derivatives—including omeprazole—belong to the group of irreversible gastric H^+/K^+ ATPase inhibitors, and they are clinically effective against acid-related diseases. They bind to sulfhydryl group(s) in the enzyme, resulting in a covalent linkage between the compound and the enzyme (Lorentzon et al., 1985). Due to the irreversibility of the enzyme inactivation, benzimidazole derivatives exhibit a powerful and long-lasting inhibition of the secretion of gastric acid. However, they may have several disadvantages in clinical practice, such as bacterial overgrowth resulting from extremely long-lasting anacidity (Wingate, 1990; Larner and Lendrum, 1992), and possible carcinogeneicity due to hypergastrinemia (Carlsson et al., 1986).

Reversible types of H^+/K^+ ATPase inhibitors include 2-methyl-8-(phenyl-methoxy)-imidazo-1,2-pyridine 3-

* Corresponding author. Tel.: +82-42-860-7542; fax: +82-42-861-4246.
E-mail address: hgcheon@pado.krict.re.kr (H.G. Cheon).

0014-2999/01/$ - see front matter
PII: S0014-2999(00)00919-5

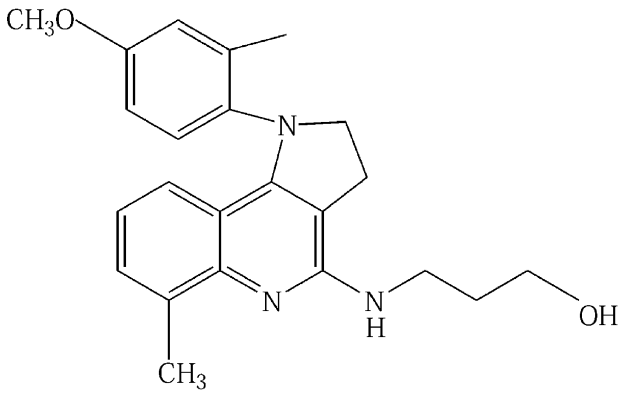

Fig. 1. Chemical structure of DBM-819.

acetonitrile (SCH28080) and 3-butylyl-8-methoxy-4-(2-tolylamino)quinoline (SK and F96067), and some of them have been tested in clinical trials (Pope and Parsons, 1993). Reversible H^+/K^+ ATPase inhibitors are expected to have a similar effectiveness as the irreversible compounds, but because they produce a more reproducible and shorter-lived effect, they allow greater dosing flexibility. In order to develop a new reversible H^+/K^+ ATPase inhibitor, 1-(2-methyl-4-methoxyphenyl)-4-[(3-hydroxypropyl)amino]-6-methyl-2,3-dihydropyrrolo[3,2-*c*]quinoline (DBM-819) was synthesized in our laboratory and examined for its biochemical properties. The chemical structure of DBM-819 is shown in Fig. 1.

2. Materials and methods

2.1. Materials

Adenosine 5'-triphosphate (Na_2ATP, disodium salt), nigericin, trizma base (Tris), trichloroacetic acid, 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), Na^+/K^+ ATPase (dog kidney) and sucrose were obtained from Sigma (St. Louis, MO, USA). Dithiothreitol and Bio Rad dye reagent were obtained from BioRad Laboratories (Richmond, CA, USA). PD-10 columns (1.5×5 cm), prepacked with Sephadex G-25M, were obtained from Pharmacia Chemical (Piscataway, NJ, USA). Amicon-30 concentrators were obtained from Amicon (Danvers, MA, USA). Perchloric acid ($HClO_4$, 60%) was obtained from Junsei Chemical (Tokyo, Japan). Butylacetate was obtained from Showa Chemical (Tokyo, Japan). DBM-819 was synthesized in Dongbu Hannong Chemical (Taejon, Korea). Omeprazole [5-methoxy-2-(4-methoxy-3,5-dimethyl-2-pyridinyl)-methylsulphinyl-1*H*-benzimidazole] was used as a reference compound.

2.2. Preparation of the gastric microsomal fraction containing the H^+/K^+ ATPase

The fundic mucosae of New Zealand White Rabbits (2–3 kg) were scraped off and homogenized in 40 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose, 2 mM HEPES, 2 mM $MgCl_2$, and 2 mM EDTA. The homogenate was centrifuged for 30 min at $10{,}000 \times g$, and the resulting supernatant was subsequently centrifuged for 60 min at $100{,}000 \times g$. The pellets were resuspended in a minimum volume of 40 mM Tris/HCl buffer (pH 7.4) and stored at −70°C until used. The protein concentration of the preparation was determined using the method of Bradford (1976), with bovine serum albumin as the standard.

2.3. H^+/K^+ ATPase assay

The reaction mixture (200 μl) contained the enzyme preparation (25 μg) in 40 mM Tris/HCl, pH 7.4, 4 mM $MgCl_2$, 5 μg/ml nigericin in methanol, with or without 48 mM KCl and 6 mM NH_4Cl. DBM-819 was dissolved in DMSO and pre-incubated with the enzyme preparation for 30 min at 37°C. The final concentration (2%) of DMSO in the reaction mixture did not affect the enzyme activity. After the reaction was started 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 trichloroacetic acid (50 μl) and then centrifuged. The amount of inorganic phosphate released from Na_2ATP in the supernatant was determined spectrophotometrically, according to Yoda and Hokin (1970). Specific H^+/K^+ ATPase activity was determined by subtracting the basal enzyme activity measured in the absence of KCl and NH_4Cl. Inhibition was calculated as the percent inhibition against maximal stimulation, and IC_{50} values were obtained from a typical dose–response curve. The assay medium contained 2% methanol, which did not affect the enzyme activity.

2.4. Na^+/K^+ ATPase assay

The reaction mixture contained 2 mM $MgCl_2$, 2 mM Na_2ATP, 40 mM Tris/HCl, pH 7.4, and 20 μg Na^+/K^+ ATPase (from Sigma), with or without 100 mM NaCl and 10 mM KCl (to give 245 μl total volume). The reaction was started by adding DBM-819 in DMSO (5 μl). After a 30-min incubation at 37°C, the reaction was stopped by adding 30% cold trichloroacetic acid. The enzyme activity was assayed by measuring the amount of inorganic phosphate released from ATP, according to Yoda and Hokin (1970).

2.5. Reversibility of the DBM-819-induced inactivation of H^+/K^+ ATPase activity

The enzyme preparation (0.25 mg/ml) was incubated with either DMSO alone, DBM-819 (50 or 100 μM) in

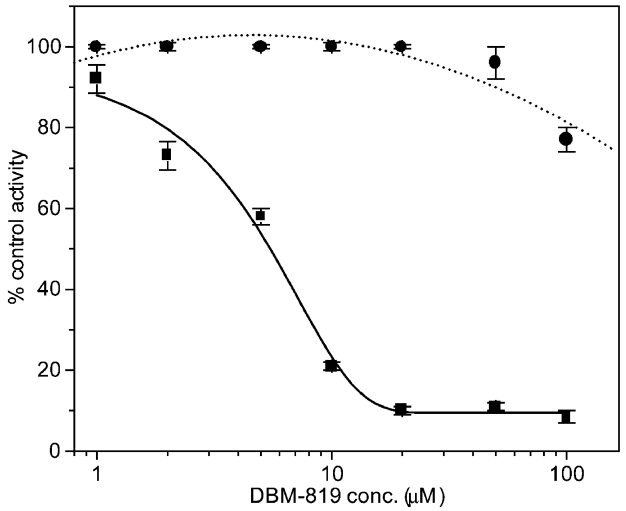

Fig. 2. Effects of DBM-819 on H^+/K^+ ATPase and Na^+/K^+ ATPase activities. Either H^+/K^+ ATPase (■; 25 μg) or Na^+/K^+ ATPase (●; 20 μg) was treated with various concentrations of DBM-819, and the remaining activity was determined 30 min later as described in Section 2. Results are expressed as percent control activity (enzyme incubated with DMSO alone). Data represents means ± S.E. of three separate experiments.

DMSO, or omeprazole (400 μM) for 10 min at 37°C. The final concentration of DMSO in the incubation mixture was 2%, which did not affect control activity. An aliquot (50 μl) was taken out at the end of the incubation, and H^+/K^+ ATPase activity was assayed as described above. The remaining portion (200 μl) of the incubation mixture was passed through a PD-10 desalting column, and the eluant containing the enzyme preparation was collected and concentrated using an Amicon-30 concentrator. The protein concentration of the eluant was determined and adjusted for H^+/K^+ ATPase assay. The enzyme activity was determined as described above.

2.6. K+ kinetics experiments

To investigate the kinetic properties of DBM-819-mediated inhibition, gastric H^+/K^+ ATPase activity was measured over a range of KCl concentrations (0.8–24 mM) in the presence of DBM-819 (1–6 μM).

2.7. In vitro studies of DBM-819

The ability of dithiothreitol to protect H^+/K^+ ATPase against inactivation by DBM-819 was tested. The H^+/K^+ ATPase preparation was pre-incubated with dithiothreitol (0.5 or 1 mM) at 37°C for 5 min, followed by incubation with 10 μM DBM-819. After 30 min, H^+/K^+ ATPase activity was assayed as described above. To examine the effect of the acidification of DBM-819 on the inactivation of H^+/K^+ ATPase activity, DBM-819 (100 μM) was incubated at 37°C for 20 min in 40 mM Tris/HCl buffer of pH 5.2, pH 6.2, or pH 7.4. Each solution was then added to the incubation mixture containing the gastric H^+/K^+ ATPase preparation, which resulted in a 50-fold dilution of DBM-819. The assay mixture was then incubated for 30 min at 37°C and assayed for the remaining H^+/K^+ ATPase activity.

2.8. Statistical analysis

All values shown in the figures and tables represent the means ± S.E. IC_{50} values with 95% confidence limits were estimated from the linear regression analysis of the log dose versus percent activity (relative to control) curve. Statistical evaluation of the results was performed by analysis of variance followed by Dunnett's test, and by the unpaired Student's *t*-test. A P value of less than 0.05 was regarded as statistically significant.

3. Results

3.1. Concentration-dependent inactivation of H^+/K^+ ATPase by DBM-819

DBM-819 caused a concentration-dependent loss of H^+/K^+ ATPase activity, as shown in Fig. 2. The IC_{50} value was estimated to be 5.0 ± 0.65 μM, whereas that of omeprazole was 22.4 ± 1.80 μM. In addition, the inhibitory effect of DBM-819 on Na^+/K^+ ATPase—a related ATPase—was very weak (IC_{50} > 100 μM), despite the similarity of the amino acid sequences of these two ATPases. These results suggest that DBM-819 is a selective and potent inhibitor of gastric H^+/K^+ ATPase.

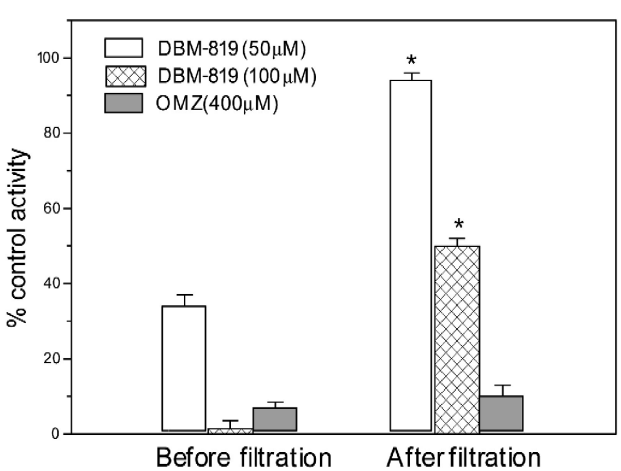

Fig. 3. Reversibility of the DBM-819-induced inactivation of H^+/K^+ ATPase activity. H^+/K^+ ATPase (62.5 μg) was incubated with either DBM-819 (50, 100 μM) or omeprazole (400 μM) for 10 min. Enzyme activity was determined before and after filtration of the incubation mixture through a Sephadex G-25M column. Results represent means ± S.E. of three separate experiments. * $P < 0.05$ vs before filtration.

3.2. Reversibility of the DBM-819-induced inactivation of H^+/K^+ ATPase activity

The reversibility of DBM-819-mediated inhibition of H^+/K^+ ATPase was investigated by filtration of the reaction mixture through a Sephadex G-25M column. As shown in Fig. 3, gastric H^+/K^+ ATPase activity was completely restored after filtration when the activity had been inhibited by the lower concentration of DBM-819 (50 μM). Inactivation of H^+/K^+ ATPase activity by the higher concentration of DBM-819 (100 μM) resulted in the partial recovery of activity after filtration. As a negative control, omeprazole-treated enzyme mixture (known for the irreversible nature of the inactivation) was passed through the Sephadex G-25M column, but there was hardly any enzyme activity in the eluant. These results suggest that DBM-819 may inhibit gastric H^+/K^+ ATPase in a reversible manner.

3.3. Kinetics of the DBM-819-mediated inactivation of H^+/K^+ ATPase activity

The mechanism of the inhibition of H^+/K^+ ATPase activity by DBM-819 was investigated by measuring the inhibitory effect of DBM-819 on enzyme activity in the presence of various concentrations of the activating cation K^+ (Fig. 4). The results were fitted best by assuming a non-competitive pattern of inhibition, with an estimated K_i value of 2.75 ± 0.48 μM.

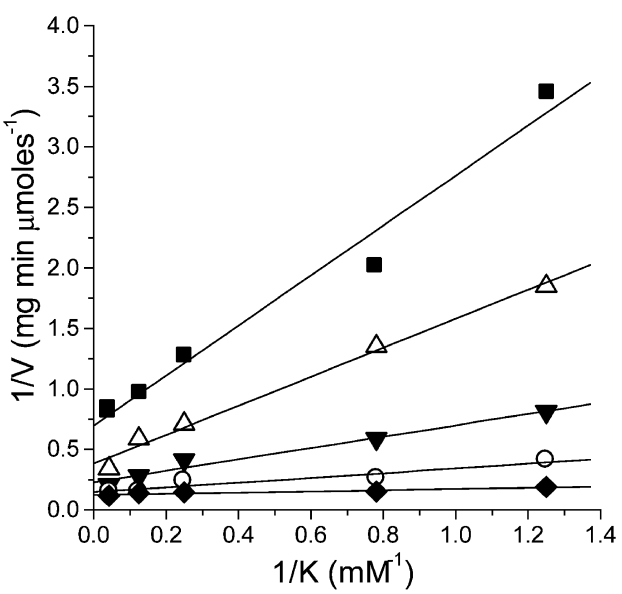

Fig. 4. Lineweaver–Burk plot of the kinetics of K^+-noncompetitive inhibition of H^+/K^+ ATPase activity. Assays were performed in the presence of 0.8–24 mM KCl and 0 (♦), 1 (○), 2 (▼), 4 (△), 6 (■) μM DBM-819. Experimental details are described in Section 2. Data shown are from a single representative experiment.

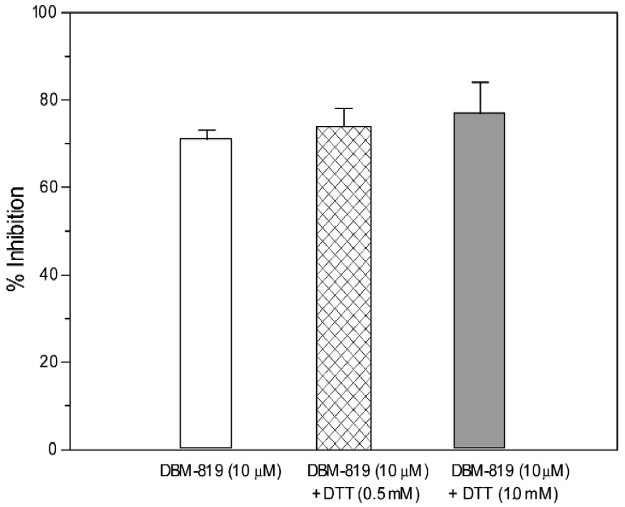

Fig. 5. Effect of dithiothreitol on the DBM-819-mediated inhibition of H^+/K^+ ATPase activity. H^+/K^+ ATPase preparation was preincubated with dithiothreitol (0.5, 1.0 mM) at 37°C for 5 min. DBM-819 (10 μM) was then added to the preincubation mixture and further incubated for 30 min. The H^+/K^+ ATPase activity was determined as described in Section 2. Results are expressed as means ± S.E. of three separate experiments.

3.4. Protection of the DBM-819-mediated inactivation of H^+/K^+ ATPase activity

The effect of the sulfhydryl reducing agent dithiothreitol on the DBM-819-mediated inhibition of H^+/K^+ ATPase activity was investigated. As shown in Fig. 5, addition of either 0.5 or 1 mM dithiothreitol to the incubation mixture—prior to the addition of 10 μM DBM-819—did not protect the H^+/K^+ ATPase from inactivation by DBM-819. This observation suggests that the inactivation by DBM-819 does not involve the essential cysteine residue(s) of the enzyme.

3.5. Effect of acidification of DBM-819 on the inactivation of H^+/K^+ ATPase activity

Since omeprazole appears to be more potent in inhibiting H^+/K^+ ATPase at acidic pH values (Keeling et al., 1985), the effect of acid exposure of DBM-819 on its H^+/K^+ ATPase inhibitory action was studied. As shown in Table 1, incubation of DBM-819 under weak acidic

Table 1
Effect of transient acidification of DBM-819 on its inhibitory action on gastric H^+/K^+ ATPase[a]

pH condition	Percent inhibition
7.4	18 ± 2.0
6.2	49 ± 3.2
5.2	62 ± 1.3

[a]After pretreatment of DBM-819 (100 μM) 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 DBM-819. Values are the means ± S.E. of three separate experiments.

conditions resulted in a more effective inactivation of H^+/K^+ ATPase activity. Thus, it seems that some form of acid activation of DBM-819 occurs in the inhibition process.

4. Discussion

Reversible gastric H^+/K^+ ATPase inhibitors have recently been proposed as important therapeutic candidates for peptic ulcer diseases (Pope and Parsons, 1993). In attempting to develop reversible gastric H^+/K^+ ATPase inhibitors for novel anti-ulcer agents in our laboratory, we chose DBM-819 as a promising compound. In an in vitro system, DBM-819 exhibited potent inactivation of rabbit gastric H^+/K^+ ATPase, being superior to omeprazole, which was the reference compound. Furthermore, DBM-819 was shown to have a greater than 20-fold selectivity for the gastric H^+/K^+ ATPase, relative to that for the closely related Na^+/K^+ ATPase. Consistent with previous results (Lorentzon et al., 1985), the inhibition of the H^+/K^+ ATPase by omeprazole was not reversible by filtration of the incubation mixture through a Sephadex G-25M column. In contrast to the irreversible action of omeprazole, DBM-819 was shown to be a reversible H^+/K^+ ATPase inhibitor. In addition, the kinetics of inhibition revealed that DBM-819 was a non-competitive inhibitor of H^+/K^+ ATPase activity with respect to the activating cation K^+.

The reversibility of the enzyme inhibition may confer several clinical benefits of DBM-819 over covalent H^+/K^+ ATPase inhibitors such as omeprazole. These include better control of dosing, and fewer problems with bacterial overgrowth, hypergastrinemia and carcinoid lesions in the stomach. In fact, long-term administration of omeprazole has been shown to induce the development of gastric carcinoma in experimental animals (Ekman et al., 1985; Tielemans et al., 1989). In addition, the recovery from complete acid suppression by irreversible inhibitors is quite slow, since it depends on de novo synthesis of the enzyme. It is therefore not surprising that some reversible H^+/K^+ ATPase inhibitors are currently under clinical development for therapeutic use.

It has been reported that omeprazole, a prototype of substituted benzimidazole derivatives, is activated to a reactive intermediate under acidic conditions before binding covalently to essential sulfhydryl group(s) in the H^+/K^+ ATPase (Keeling et al., 1985). Similar to published data on omeprazole, the present study showed that the efficacy of DBM-819 in inactivating H^+/K^+ ATPase activity was correlated to the acidity of the pre-incubation environment. The pre-incubation of DBM-819 under acidic conditions resulted in a more effective inactivation of H^+/K^+ ATPase activity, as compared with the inhibition achieved at neutral pH. This result is suggestive of the activation of DBM-819 under acidic conditions, although the chemical nature of the activated form is unknown. Alternatively, DBM-819 may bind to the H^+/K^+ ATPase more effectively at acidic pH.

Previous reports have indicated that omeprazole binds to cysteine 813 in the fifth to sixth transmembrane segments, and this finding correlates with the inhibition of acid secretion (Besancon et al., 1997). Correspondingly, the presence of dithiothreitol as well as β-mercaptoethanol has been shown to abolish the inhibitory effect of omeprazole (Lorentzon et al., 1985). Contrary to the results for omeprazole, the present study found that DBM-819-mediated inhibition of H^+/K^+ ATPase was not prevented by pre-incubation with dithiothreitol. Therefore, DBM-819 appears to inhibit the gastric H^+/K^+ ATPase by a different mechanism from that of omeprazole. Based on the present results, the mechanism of action of DBM-819 may not involve the covalent modification of key sulfhydryl group(s) in the enzyme.

In summary, the present findings show that DBM-819 is a potent, selective and reversible inhibitor of gastric H^+/K^+ ATPase, although the exact mechanism of the inactivation process cannot be defined with certainty. The high selectivity and reversibility of DBM-819 may add to its benefits as a therapeutic agent. Based on its present in vitro properties, DBM-819 is worthy of in vivo investigation for its anti-secretory and anti-ulcer activity. An accompanying paper shows that DBM-819 is an effective anti-ulcer agent in vivo.

References

Besancon, M., Simon, A., Sachs, G., Shin, J.M., 1997. Sites of reaction of the gastric H^+/K^+ ATPase with extracytoplasmic thiol reagents. J. Biol. Chem. 272, 22438–22446.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Carlsson, E., Larsson, H., Mattsson, H., Ryberg, B., Sundell, G., 1986. Pharmacology and toxicology of omeprazole—with special reference to the effects on the gastric mucosa. Scand. J. Gastroenterol. 21 (118), 31–38, Suppl.

Ekman, L., Hansson, E., Havu, N., Carlsson, E., Lundberg, C., 1985. Toxicological studies on omerpazole. Scand. J. Gastroenterol. 20 (108), 53–69, Suppl.

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

Larner, A.J., Lendrum, R., 1992. Oesophageal candidasis after omeprazole therapy. Gut 33, 860–861.

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

Maeda, M., Oshiman, K.I., Tamura, S., Futai, M., 1990. Human gastric H^+/K^+ ATPase gene. Similarity to Na^+/K^+ ATPase genes in exon/intron organization but difference in control region. J. Biol. Chem. 265, 9027–9032.

Pope, A.J., Parsons, M.E., 1993. Reversible inhibitors of the gastric H^+/K^+ transporting ATPase: a new class of antisecretory agent. Trends Pharmacol. Sci. 14, 323–325.

Sachs, G., Chang, H.H., Rabon, E., Schackman, R., Lewin, M., Saccomani, G., 1976. A nonelectrogenic H^+ pump in plasma membranes of hog stomach. J. Biol. Chem. 251, 7690–7698.

Shin, J., Besancon, M., Bamberg, K., Sachs, G., 1997. Structural aspects of the gastric H^+/K^+ ATPase. Ann. N. Y. Acad. Sci. 834, 65–76.

Shull, G.E., 1990. cDNA cloning of the β-subunit of the rat gastric H,K-ATPase. J. Biol. Chem. 265, 12123–12126.

Shull, G.E., Lingrel, J.B., 1986. Molecular cloning of the rat stomach H^+/K^+ ATPase. J. Biol. Chem. 261, 16788–16792.

Tielemans, Y., Hakanson, R., Sundler, F., Willems, G., 1989. Proliferation of enterochromaffin-like cells in omeprazole-treated hypergastrinemic rats. Gastroenterology 96, 723–729.

Wingate, D.L., 1990. Acid reduction and recurrent enteritis. Lancet 335, 222.

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