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Inhibitory Action of YJA20379, a New Proton Pump Inhibitor on *Helicobacter pylori* Growth and Urease

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The activities of two types of antiulcer agents against 9 strains of *Helicobacter pylori* (*H. pylori*) were determined by the agar dilution method. The antiulcer agents were YJA20379, a newly synthesized proton pump inhibitor developed by Yung-Jin Pharmaceutical company, and omeprazole. Both compounds were found to have significant activities against this organism. The MIC values of YJA20379 and omeprazole were 11.7 and 31.25 μg/ml, respectively. In addition, the inhibitory potency of both compounds was investigated on *H. pylori* urease which is believed to be an important colonization and virulence factor in the pathogenesis of gastritis and peptic ulcers. These compounds dose-dependently inhibited urease extracted with distilled water and their IC_{50} values were 16.4×10^{-5} M and 14.3×10^{-5} M, respectively. In addition, a pH-dependent study to determine whether inhibitory potency would be activated by acid condition was performed. It was found that unlike omeprazole, YJA20379 was not affected by acid condition. To determine the inhibition pattern and optimal concentration of substrate, kinetics were evaluated at various pH levels (pH 5.0, 7.0, and 8.5). The data show that YJA20379 noncompetitively inhibited *H. pylori* urease and K_M/K_i values were 0.96 mM/60 μM (pH 5.0), 0.56 mM/141.5 μM (pH 7.0), and 1.94 mM/34 μM (pH 8.5), respectively. Based on data obtained, it is concluded that YJA20379 is a significant inhibitor of *H. pylori* growth and urease and therefore, taking these results into consideration, YJA20379 might be a beneficial therapy for gastritis and peptic ulcers induced by *H. pylori*.

Key words : YJA20379, Omeprazole, *Helicobacter pylori*, Agar dilution method, Urease

INTRODUCTION

Since a microaerophilic Gram (-), spiral or curved bacterium, *Helicobacter pylori* (*H. pylori*) (Goodwin, 1989) was first isolated and cultured by Warren and Marshall in 1983 (Warren and Marshall, 1983), numerous reports have shown that the distribution of *H. pylori* is highly associated with both gastritis and peptic ulcers (Blaser, 1990; Graham, 1989; Marshall, 1990; Marshall and Warren, 1984; Wyatt and Dixon, 1988). There are also several reports indicating that eradication of *H. pylori* with antiulcer agent-antibiotic combinations leads to a persistent improvement of gastritis and a lower relapse rate of peptic ulcer (Eberhardt and Kasper, 1990; Humphreys *et al.*, 1988; Marshall *et al.*, 1987; Marshall *et al.*, 1988; McNulty *et al.*, 1986; Rauws *et al.*, 1988; Rauws and Tytagt, 1990). Thus, it is now widely accepted that *H. pylori* is involved in the pathogenesis of peptic ulcers and the development of gastric carcinoma. Taking a close association between *H. pylori* and gastroduodenal disorders into consideration, the effects of YJA20379 and omeprazole have been examined on this organism's growth by the agar dilution method and found that YJA20379 had more potent activity against *H. pylori* growth than that of omeprazole. *H. pylori* is also characterized by strong urease activity which hydrolyzes urea in gastric juice to produce ammonia and carbon dioxide. Ammonia generated by urease elevates the level of pH in the stomach and protects this acid-sensitive bacterium (Lee *et al.*, 1993). Furthermore, high concentrations of ammonia inhibit the consumption of oxygen and reduce the production of ATP in the gastric epithelial cells and in the mitochondria (Tsujii *et al.*, 1992). In addition, the fact that urease is highly homologous among *Helicobacter* species suggests that urease plays a critical role in the survival of these or-

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ganisms in the gastric mucosa and in the pathogenesis of the diseases caused by *H. pylori*. The aim of this present study was to determine whether YJA20379, a newly synthesized benzothiazole-derivative and antiulcer agent possesses the advantages combined properties of being a specific and potent inhibitor of *H. pylori* urease as well as growth. The effects of omeprazole were also examined for comparison.

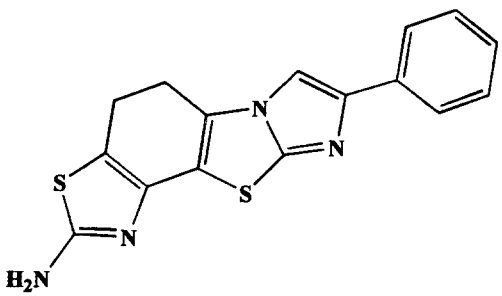
MATERIALS AND METHODS

Antiulcer agents and urease inhibitors

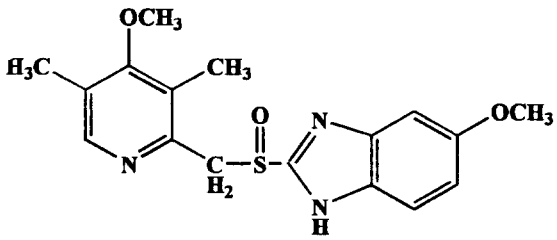
YJA20379, 2-amino-4,5-dihydro-8-phenylimidazo [2,1-b] thiazole [5,4-g] benzothiazole, and amoxicilin were synthesized by Yung-Jin Pharmaceutical Company and omeprazole was purchased by Chong-Kun-Dang Pharmaceutical Company (Fig. 1). These compounds were dissolved in dimethyl sulfoxide (DMSO) at appropriate concentration and then diluted in either distilled water (DW), 20 mM citrate buffer or 20 mM phosphate buffer to achieve the desired concentrations.

Bacterial strains and growth conditions

H. pylori NCTC 11637 (type strain), 11638, 12385, and 11916 were purchased by National Collection of Type Cultures. Clinical isolates were kindly provided by Dr. Young-Chil Ha, Seoul National University, Seoul, Korea. The identification of *H. pylori* strains was based on standard biochemical tests (Marshall *et al.*, 1984). Frozen stocks were prepared from overnight cultures of *H. pylori* grown in Brucella broth (BBL, U.S.A.) containing 5% FBS, with shaking at 130 rpm on a rotatory shaker in a sealed jar under microaerobic conditions. *H. pylori* from this overnight culture was stored in liquid nitrogen tank with 15% glycerol and 10% FBS.



YJA20379



OMEPRAZOLE

Fig. 1. Chemical structures of YJA20379 and omeprazole.

A fresh culture was prepared by directly plating the frozen stock on blood agar base #2. Plates were incubated for 4~5 days at 37°C in a microaerobic atmosphere. Colonies collected from plates were incubated in 250 ml brucella broth supplemented with 5% FBS in a 1000 ml flask for culturing *H. pylori* in large quantities. The culture flasks were incubated with shaking at 130 rpm in a microaerobic atmosphere for 3 days. Purity control was carried out at each stage of growth by examination with Gram staining, nitrate and nitrite reduction, urease, alkaline phosphatase and catalase test, and hippurate hydrolysis.

Determination of MICs by the agar dilution method

H. pylori strains were grown on blood agar base #2 supplemented with 5% FBS at 37°C for 3~5 days in microaerobic conditions and suspended in brucella broth with 5% FBS to give the turbidity equivalent to McFarland standard No. 0.5; this resulted in suspensions containing about 5×10^8 CFU/ml. The bacterial suspensions were applied to the blood agar base #2 plates containing two-fold serial dilutions of antiulcer agents by a multi point inoculation capable of delivering 1 μl samples. The plates were incubated at 37°C in a microaerobic environments. After 3 days of incubation, there was satisfactory growth of all strains tested on the control plates. Readings were performed after 3 days unless otherwise specified. Minimum Inhibitory Concentrations (MICs) were defined as the lowest concentrations of the test compounds inhibiting visible bacterial growth.

Preparation of urease

The cells were harvested by centrifugation (20,000× g for 20 min at 4°C), washed twice with 20 mM sodium citrate buffer (pH 5.0), 20 mM sodium phosphate buffer (pH 7.0), or 20 mM triethanolamine buffer (pH 8.5), and then resuspended in the same buffers. The resuspended cells were disrupted by sonication and centrifuged at 20,000×g for 20 min at 4°C. The supernatant was used for the measurement of cell-free urease activity.

Urease assay and inhibition

Reaction mixtures composed of 100 μl enzyme solution and 300 μl buffer at different pH values (5.0, 7.0, and 8.5) containing 100 mM urea were incubated at 37°C for 30 min, after which 100 μl 1N H_2SO_4

was added. The buffers used in this assay were 20 mM sodium citrate buffer (pH 5.0), 20 mM sodium phosphate buffer (pH 7.0), and 20 mM triethanolamine buffer (pH 8.5). For ammonia determination using the indophenol method (Giasti and Galanti, 1974), 2.5 ml each of phenol-nitroprusside reagent and alkali reagents were added to the reaction mixture. After incubation at 65°C for 20 min, the absorbance at 630 nm was measured.

In order to determine the IC_{50} (concentration producing 50% inhibition), mixtures of 50 μl each of enzyme solution and YJA20379 solution at various concentrations were preincubated for 15 min at 37°C, after which the urease activities were measured by the indophenol method as described above.

Urease activity was estimated with jack bean urease (Sigma Chemicals) as a standard, the specific activity of which was 5.7 units/mg protein. One unit of urease activity indicates 1 μmole of ammonia liberated per min at 25°C. Percent inhibition was determined by the following equation: % inhibition=[(activity without inhibitors-activity with inhibitors)/activity without inhibitors]×100.

The amounts of protein are determined by a modification of a procedure described by Lowry (Markwell *et al.*, 1981).

pH-dependent study

In order to determine whether inhibitory potency would be affected by acidic pH (pH 5.0), mixtures of 50 μl each of enzyme solution and YJA20379 at pH 5.0 and 7.0 were preincubated for 15 min at 37°C, after which the urease activities were measured by the indophenol method.

Enzyme kinetics

In order to determine inhibitory pattern, K_M and K_i values, mixtures of 50 μl each of enzyme solution and YJA20379 at various concentrations of urea were incubated for 30 min at 37°C, after which the urease activities were measured by the indophenol method.

RESULTS

Strains of *H. pyori*

All of the bacteria were confirmed to be Gram (-), slender curved or spiral rods. As shown Table I, all of the 5 clinical isolates tested were found to share identical characteristics with those found in 4 reference strains including the nomenclature type strain NCTC 11637. They failed to grow aerobically, but did grow microaerobically. They were found positive for catalase, alkaline phosphatase, and urease. But they reduced neither nitrate nor nitrite. All of the strains tested were negative for hippurate hydrolysis.

Activities of antiulcer agents against *H. pylori*

The MICs of 3 agents (Omeprazole, YJA20379, and amoxicillin) for 9 strains of *H. pylori* were determined by the agar dilution method. Table II showed the ranges of MICs and the concentrations required to inhibit 50% and 90% of the strains, which were determined after 3 days of incubation. All of the plates for the MIC tests were further incubated for 2 additional days, but there were no change in the MICs.

Though less active than amoxicillin, the two proton pump inhibitors, YJA20379 and omeprazole showed notable activities. The activity of YJA20379 was at least three times more potent than that of omeprazole.

Effect of YJA20379 and omeprazole on the *H. pylori* urease

Both YJA20379 and omeprazole inhibited *H. pylori*

Table II. Activities of various antiulcer agents against of *H. pylori* strains

Agents	MIC (μg/ml)[a]		
	Range	50%	90%
Omeprazole	1.9~250	15.6	31.25
YJA20379	0.75~250	11.7	11.7
Amoxicillin	0.0125~12.5	0.05	0.05

[a]MICs were determined by the agar dilution method on blood agar

Table I. Biochemical characteristics of 9 strains of *H. pylori*

Characteristics	NCTC				Clinical Isolates				
	11637[a]	11638	11916	12385	4	11	43	82516	82548
Aerobic growth	-	-	-	-	-	-	-	-	-
Microaerobic growth	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-
Nitrite reduction	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+
Alkaline phosphatase	+	+	+	+	+	+	+	+	+
Hippurate hydrolysis	-	-	-	-	-	-	-	-	-

[a]Type strain, +: Positive, -: Negative

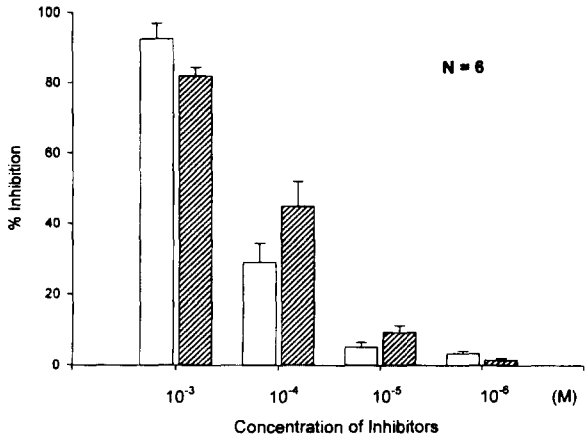


Fig. 2. Inhibitory effects of YJA20379 and omeprazole on *H. pylori* urease activity at pH 7.0 (■: YJA20379, ▨: Omeprazole).

Table III. Inhibitory effects of YJA20379 and omeprazole on *H. pylori* urease activity at pH 5.0 and 7.0

	IC_{50} (10^{-5} M)	
	YJA20379	Omeprazole
pH 5.0	22.4	0.7
pH 7.0	16.4	14.3

urease activity in a concentration-dependent manner at pH 7.0 when preincubated for 15 min at 37°C (Fig. 2).

Table III showed that the IC_{50} values of YJA20379 and omeprazole at pH 5.0 and 7.0 were 22.4/16.4× 10^{-5} M and 0.7/14.3× 10^{-5} M, respectively.

Effect of YJA20379 and omeprazole by acidic pH on the *H. pylori* urease

At pH 5.0, omeprazole was the more effective inhibitor compared with YJA20379 which reduced little effect except at high concentrations (Fig. 3).

Kinetic study of YJA20379 on the *H. pylori* urease

Lineweaver-Burk plots for the inhibition of *H. pylori* urease activity by YJA20379 at different pH values are shown in Fig. 4. YJA20379 inhibited urease in a noncompetitive manner at all pH values ranging from 5.0 to 8.5.

K_M/K_i values determined for YJA20379 were 0.96 mM/60 μM (pH 5.0), 0.56 mM/141.5 μM (pH 7.0), and 1.94 mM/34 μM (pH 8.5) (Table IV).

DISCUSSION

In the present study, two antiulcer agents with the ability to inhibit the proton pump mechanisms in gastric parietal cells (Sohn *et al.*, unpublished data), YJA 20379 and omeprazole, showed marked *in vitro* activities against *H. pylori* growth. In particular, YJA20379

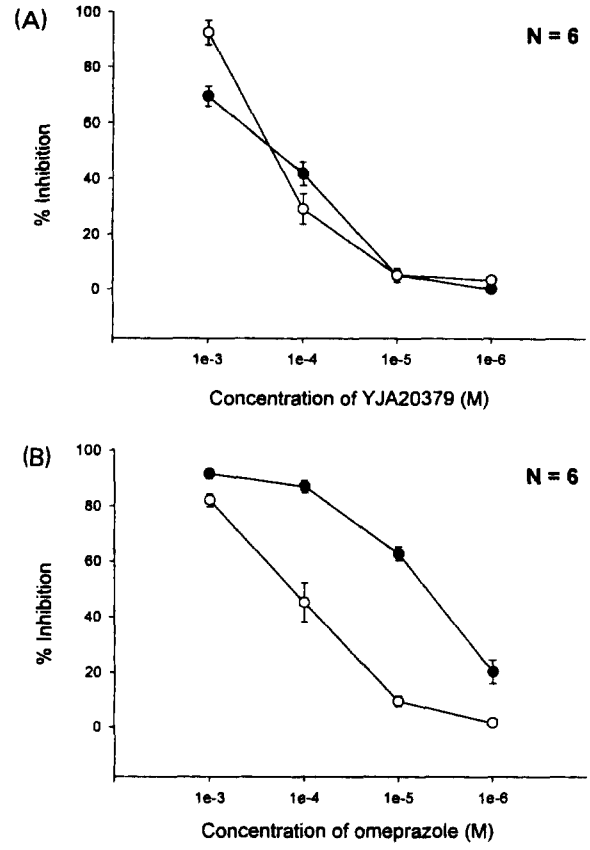


Fig. 3. Inhibitory effects of (A) YJA20379 and (B) omeprazole on *H. pylori* urease activity at pH 5.0 and 7.0 (●—●: pH 5, ○—○: pH 7).

was at least three times more potent than omeprazole and its *in vitro* activity was comparable to that of bismuth salts, which are used as antiulcer agents and are known to have antibacterial activity against this organism. There are also a number of antibiotics known to be highly active *in vitro* against *H. pylori* (Amstrong *et al.*, 1987; Goodwin *et al.*, 1986; Lambert *et al.*, 1986). However, when tested as a single agent in clinical therapy, there are few drugs which are able to successfully eradicate *H. pylori*. So far, the best results in *H. pylori* therapy have been achieved with the combination of a non-absorbed agent with topical activity, a well-absorbed agent with systemic activity and a proton pump inhibitor (Tytgat, 1996). On the basis of these findings, it is suggested that ideal anti-*H. pylori* agents would be both locally and systemically effective against this bacterium, acid-stable and able to penetrate the mucus and crypts where *H. pylori* is found. To determine whether YJA20379 fulfilled these criteria, *in vivo* experiments have been performed to assess the effect on *H. pylori* where the test material was administered alone. Based on the results obtained, the type of combination with other drugs such as antibiotics, proton pump inhibitors, and *etc*

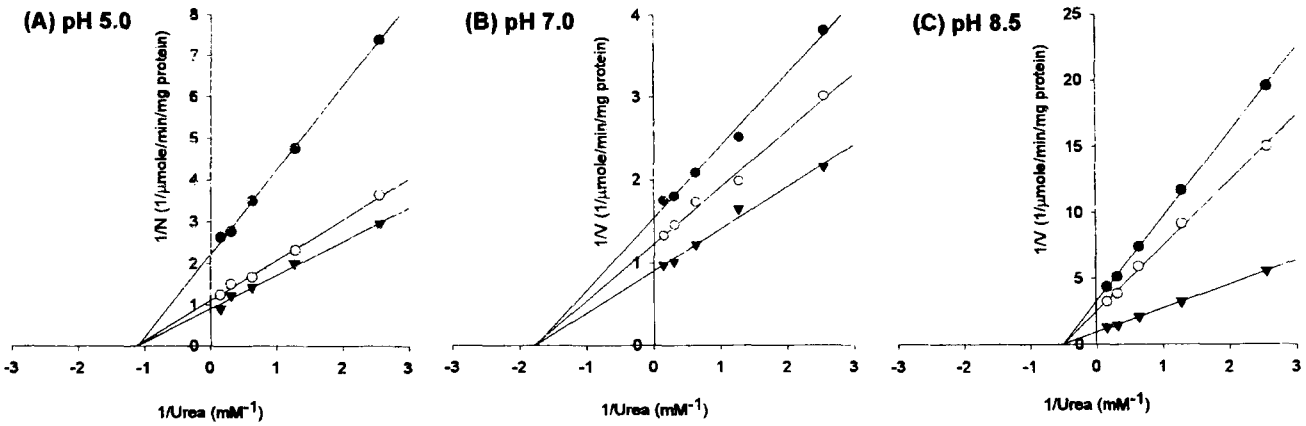


Fig. 4. Lineweaver-Burk plots for inhibition of *H. pylori* urease by YJA20379 at (A) pH 5.0, (B) 7.0, and (C) 8.5. ●—●: YJA 20379 0.1 mM, ○—○: YJA20379 0.05 mM, ▼—▼: No YJA20379.

Table IV. K_M and K_i values of YJA20379 on *H. pylori* urease activity a t pH 5.0, 7.0 and 8.5

	K_M (mM)			K_i (μM)		
	pH 5.0	pH 7.0	pH 8.5	pH 5.0	pH 7.0	pH 8.5
YJA20379	0.963	0.563	1.938	60	141.5	34

will be selected for further investigation.

As is generally known, one of the characteristics of *H. pylori* is the production of a potent urease. Considering that urease-deficient *H. pylori* is unable to colonize in piglet stomach, the extremely high level of *H. pylori* urease activity appears to be the main reason that *H. pylori* can colonize in the acidic gastric environment. Many histological observations suggest that *H. pylori* colonizes on the surface and foveolar regions or beneath the mucus layer of the gastric mucosa (Price, 1988). It is also suggested that *H. pylori* urease is an important virulence factor because of the production of ammonia which may contribute to development of gastritis and peptic ulceration (Kawano *et al.*, 1989; Takahashi *et al.*, 1991; Xu *et al.*, 1990). The inhibitory properties of YJA20379 and omeprazole on *H. pylori* urease were, therefore, examined and demonstrated that these two antiulcer agents inhibited DW-extracted urease in a concentration-dependent manner. Patients receiving YJA20379 therapy, therefore, may receive added benefit from the prevention of ammonia production by *H. pylori*. The results also demonstrated that YJA20379 noncompetitively inhibited the *H. pylori* urease, irrespective of pH conditions. Thus, considering that there is no urease in human, these results suggest that YJA20379 offers a notable advantage in therapy of ulcers induced by *H. pylori*.

Though further studies to substantiate this hypothesis will be required, it is concluded that YJA20379 has the ability to inhibit *H. pylori* growth through urease inhibiton as well as H^+/K^+-ATPase and, in combination with antibiotics, may exhibit favorable therapeutic benefit on the course of peptic ulcer treatment as a new antiulcer agent.

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