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Ecabet sodium, a locally acting antiulcer drug, inhibits urease activity of *Helicobacter pylori*

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Abstract

In order to clarify the mechanism of the anti-*Helicobacter pylori* action of ecabet sodium (ecabet), a locally acting antiulcer drug, we evaluated the effects of ecabet on *H. pylori* urease activity in vitro. *H. pylori* was cultured and a crude preparation of urease was made. Urea-dependent survival of *H. pylori* at acid pH was significantly inhibited by ecabet. The urease activity of intact cells and a crude enzyme preparation from *H. pylori* had two pH optima: pH 4.5–5.0 and 8.0. Ecabet (1–4 mg/ml) concentration dependently inhibited the urease activity of both preparations at pH 5.0, but there was no inhibition at pH 8.0. The enzyme activity was inhibited by ecabet gradually and was not restored by dilution, in contrast to the inhibition elicited by benzohydroxamic acid, a specific and reversible urease inhibitor. These results suggest that irreversible inhibition of *H. pylori* urease activity contributes to the anti-*H. pylori* action of ecabet.

Keywords: *Helicobacter pylori*; Urease; Ecabet sodium; Antiulcer drug

1. Introduction

Since the isolation of *Helicobacter pylori*, evidence has accumulated for the close association of *H. pylori* with gastroduodenal disorders (Warren and Marshall, 1983; Marshall and Warren, 1984; Raus and Tygat, 1990; Labenz and Borsh, 1994). *H. pylori* is now recognized to play a pathogenic role in patients with gastritis and peptic ulcer diseases (Marshall and Warren, 1984; Raus and Tygat, 1990; Labenz and Borsh, 1994). Urease is an enzyme which produces NH_3 by cleaving urea. The urease activity of *H. pylori* is the highest among the urease-producing bacterial species and is one of the most important virulence factors (Mobley et al., 1991). Urease-produced NH_3 causes not only functional and morphological damage in the gastric epithelium (Triebling et al., 1991; Kawano et al., 1991; Tsujii et al., 1992) but also enables *H. pylori* to survive in gastric acid by neutralizing the hydrogen ion around the organism (Marshall et al., 1990).

Ecabet sodium (ecabet) is marketed in Japan as an antiulcer drug. This drug inhibits peptic activity (Onoda et al., 1989; Ito et al., 1993a,b) and enhances the defensive activity of the mucosa in the stomach and duodenum (Onoda et al., 1990; Kinoshita et al., 1993). Recently, an anti-*H. pylori* action of ecabet has been reported in Japanese monkeys (Fukuda et al., 1994, 1996) and in combination therapy with antibiotics in patients with peptic ulcer (Fukuda et al., 1995; Isomoto et al., 1995). Previously we have shown that ecabet affects the survival of *H. pylori* in low pH medium in the presence of urea (Shibata et al., 1995). In the present study, we evaluated the effects of ecabet on the urease activity of *H. pylori*.

2. Materials and methods

2.1. Compounds and reagents

The disodium salt of ecabet was synthesized at the Organic Chemistry Research Laboratory, Tanabe Seiyaku, Benzohydroxamic acid and urea were purchased from

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0014-2999/98/$19.00

Sigma (St. Louis, MO, USA) and Nacalai Tesque (Kyoto, Japan), respectively. Each compound was dissolved in purified water. BCA Protein Assay Reagent™ (Pierce, Rockford, IL, USA) was used for the measurement of protein concentration.

2.2. Effects of ecabet sodium on survival of H. pylori at acidic pH

Ecabet was dissolved in 130 mM citrate buffer at pH 3.0 and preincubated for 10 min at 37°C. *H. pylori* NCTC 11637 was inoculated in brain heart infusion broth (Eiken Kagaku, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum and incubated at 37°C for 48 h in a microaerobic condition (5% O_2, 10% CO_2, and 85% N_2). After being rinsed with saline, bacterial cells were added to the buffer in the presence or absence of urea (final conc; 10 mM). After 0, 15, 30, 45 and 60 min of incubation at 37°C, an aliquot was taken and the viability of *H. pylori* was measured by the plate colony count technique (Shibata et al., 1995).

2.3. Preparation of H. pylori urease

H. pylori was cultured on Mueller–Hinton agar (Oxoid, Basingstoke, England) supplemented with 8% horse blood at 37°C for 3 days under the microaerobic conditions. Bacterial cells were collected on saline cooled with ice and suspended at a concentration of 10^5–10^7 cells per 1 ml. The bacterial cell suspension was used as a urease preparation of intact cells. A crude enzyme fraction of *H. pylori* urease was prepared from the whole cells according to a modification of the method of Dunn et al. (1990) as follows. The cell suspension was Vortex mixed for 10 min and centrifuged at $1500 \times g$ for 15 min at 4°C. The supernatant was frozen and stored in liquid nitrogen until used. The stored preparation was diluted with purified water after thawing.

H. pylori NCTC 11637 was used throughout the present study. In some experiments, clinical isolates (KC-1, 2 and 3) obtained from *H. pylori*-infected patients were also used.

2.4. Measurement of urease activity

Urease activity was assayed by measurement of the NH_3 produced in the samples. Urea was added to 50 mM citrate-HEPES buffer containing the crude enzyme or to 100 mM citrate buffer supplemented with 100 mM sucrose containing intact cells of *H. pylori* to start the enzyme reaction. After incubation at 37°C for 1 min, the reaction was stopped with 1 N sulfuric acid. After addition of 10% sodium tungstate (VI), the samples were centrifuged at $1500 \times g$ for 15 min at 20°C and the NH_3 in the supernatant was measured by the indophenol method (Akamatsu, 1952). The urease activity was expressed as mol NH_3/min per mg of enzyme or cells. Unless otherwise stated, the final concentration of urea was 10 mM, which was almost the same concentration as in gastric juice (Mobley et al., 1991). Urease activity was assayed in duplicate.

2.5. Influence of pH on urease activity of H. pylori

The solutions used were 50 mM citrate-HEPES buffers at pHs of 3.4, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. After addition of urea (250 mM) to each buffer, the mixture was preincubated at 37°C for 5 min. The crude enzyme preparation or intact cells of *H. pylori* NCTC 11637 were added to the mixture, and NH_3 in the mixture was measured as mentioned above.

2.6. Effects of ecabet and benzohydroxamic acid on urease activity

The effect of the test drug on the activity of the crude enzyme preparation was determined as follows: the test drug was added to citrate-HEPES buffer at pH 5.0, and the mixture was preincubated at 37°C for 5 min. Urea was added to the mixture, and then the urease activity was measured as mentioned above. Experiments with intact cells were carried out with 100 mM citrate buffer at pH 5.0 supplemented with 100 mM sucrose instead of citrate-HEPES buffer at pH 5.0. The enzyme activity without the test drug was used as a control to calculate the percent inhibition.

2.7. Influence of pH on the effects of ecabet and benzohydroxamic acid on urease activity

After addition of test drug to 50 mM citrate-HEPES buffers with pHs 4.4, 4.6, 4.8, and 5.0 at 37°C for 5 min, the crude enzyme preparation was added, and then the produced NH_3 in the mixture was measured as mentioned above.

2.8. Effect of preincubation time on the inhibition of urease activity by ecabet and benzohydroxamic acid

Ecabet (4 mg/ml) and benzohydroxamic acid (10 μg/ml) were added to 50 mM citrate-HEPES buffer at pH 5.0. The mixtures were incubated at 37°C for 5 min. After addition of the crude enzyme preparation of *H. pylori* NCTC 11637, the mixture was preincubated at 37°C for 1, 3, 5, 10, and 20 min. NH_3 in the mixture was measured as mentioned above.

2.9. Reversibility of urease inhibition by ecabet and benzohydroxamic acid

Reversibility of urease inhibition by ecabet and benzohydroxamic acid was measured by dilution of the reaction

mixture as follows. Ecabet (4 mg/ml) and benzohydroxamic acid (1 μg/ml) were added to citrate-HEPES buffer at pH 5.0, and the mixtures were incubated at 37°C for 5 min. After addition of the crude enzyme preparation, the mixture was incubated at 37°C for 10 min. Each mixture was diluted 10-fold with the buffer and incubated at 37°C for 30 min. After the addition of urea, the NH_3 produced was measured. NH_3 in the diluted mixture was compared with that in non-diluted mixture.

3. Results

3.1. Effects of ecabet sodium on survival of H. pylori at acidic pH

The number of viable *H. pylori* cells decreased time dependently in 130 mM citrate buffer at pH 3.0 (Fig. 1). When the buffer supplemented with 10 mM urea was used, the number of viable cells did not change (Fig. 1). The effect of urea was decreased by ecabet (0.5–2 mg/ml) in a concentration-dependent manner (Fig. 1).

3.2. The effect on urease activity of H. pylori

The urease activity of intact cells and the crude enzyme preparation over the pH range 3.4–8.5 is shown in Fig. 2. There were two optimal pH of 4.5–5.0 and 8.0 for the urease activity of both intact cells and crude enzyme of *H. pylori*.

Table 1 shows the inhibitory effect of ecabet and benzohydroxamic acid on the urease activity of the crude enzyme preparation and intact cells of *H. pylori*. Ecabet (1–4 mg/ml) depressed the urease activity of the crude enzyme preparation in a concentration-dependent manner at pH 5.0, while there was no inhibition even with 16 mg/ml at pH 8.0. Benzohydroxamic acid (0.1–10 μg/ml) inhibited the urease activity at both pH 5.0 and 8.0. There was no difference in the inhibitory potency of ecabet and benzohydroxamic acid among the strains of *H. pylori* used in the present study. The inhibition of urease activity by either ecabet or benzohydroxamic acid was slightly weaker in intact cells than in the crude enzyme preparation.

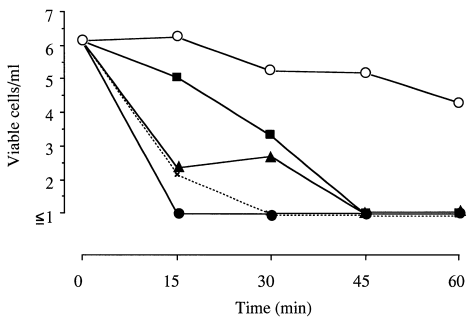

Fig. 1. Effect of ecabet sodium on the viability of *H. pylori* in pH 3.0 buffer supplemented with urea. *H. pylori* NCTC 11637 cells were exposed to ecabet sodium at concentrations of 2 (●), 1 (▲), 0.5 (■) and 0 (○) mg/ml in the presence of 10 mM urea at 0 mg/ml (×) in the absence of urea in pH 3.0 citrate buffer at 37°C. Viability was measured by the plate colony count technique.

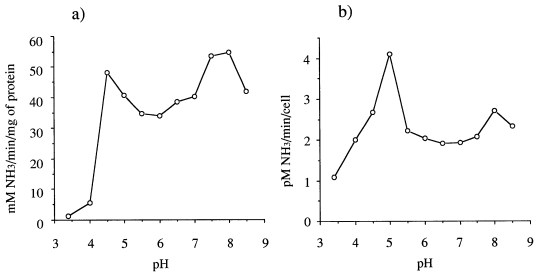

Fig. 2. Influence of pH on urease activity of *H. pylori*. Crude enzyme preparation (a) and intact cells (b) of *H. pylori* NCTC 11637 were used. The concentrations of crude enzyme preparations, cell suspension and urea were 21 μg/ml, 5×10^5 cells/ml and 250 mM, respectively.

3.3. Influence of pH on the inhibition of urease activity

As shown in Fig. 3, over the pH range of 4.4–5.0, both ecabet and benzohydroxamic acid inhibited the urease activity in pH-dependent manner: the lower the pH, the greater the inhibitory activity. While both ecabet (4 mg/ml) and benzohydroxamic acid (30 μg/ml) inhibited the urease activity at pH 5.0 to the same extent, the inhibitory activity of ecabet was greater than that of benzohydroxamic acid at pH 4.4.

Table 1
Effects of ecabet and benzohydroxamic acid on urease activity of *H. pylori* crude enzyme and intact cells

Source	Strain	pH	IC_{50}	
			Ecabet sodium (mg/ml)	Benzohydroxamic acid (μg/ml)
Crude enzyme	NCTC 11637	5.0	2.2	0.54
		8.0	>16	1.1
	KC-1	5.0	2.1	0.62
		8.0	>16	1.2
	KC-2	5.0	2.6	0.64
		8.0	>16	1.2
	KC-3	5.0	2.4	0.54
		8.0	>16	1.2
Intact cells	NCTC 11637	5.0	3.8	0.80

After ecabet (1–16 mg/ml) or benzohydroxamic acid (0.1–10 μg/ml) was preincubated with each urease source at 37°C for 10 min, the activity was measured. The activity without the drug was used as control to calculate the percent inhibition. IC_{50} was calculated by linea regression analysis. Urease activity of the crude enzyme preparation at pH 5.0 and 8.0 was 19–43 and 34–190 μM NH_3/min per mg of protein, respectively. Urease activity of viable cells was 1.5–71 μM NH_3/min per 10^6 cells.

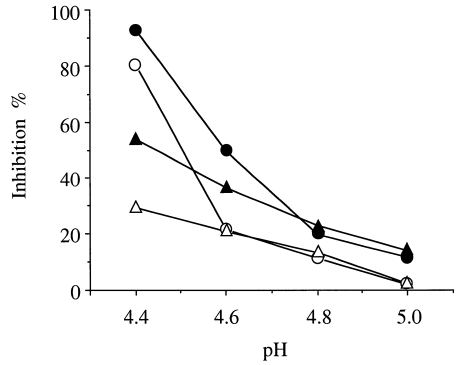

Fig. 3. Influence of pH on inhibition of urease activity by ecabet and benzohydroxamic acid. A crude enzyme preparation of *H. pylori* NCTC 11637 was added to the buffer containing the test drug and then the activity was immediately measured. The concentration of the drug was as follows. Ecabet; 2 (○), 4 (●) mg/ml. Benzohydroxamic acid; 10 (△), 30 (▲) μg/ml. Urease activities at pH 4.4, 4.6, 4.8 and 5.0 were 44, 69, 77, 77 μM NH_3/min per mg of protein, respectively.

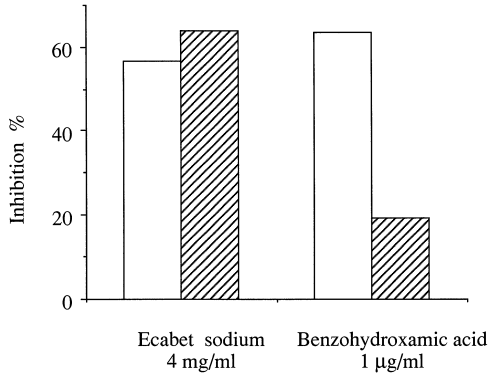

Fig. 5. Reversibility of urease inhibition by ecabet and benzohydroxamic acid. A crude enzyme preparation of *H. pylori* NCTC 11637 was added to the buffer containing the test drug (Ecabet; 4 mg/ml, benzohydroxamic acid; 1 μg/ml) and incubated at 37°C for 10 min. The incubation mixture was diluted 10-fold and the urease activity was measured. Open column represents the inhibition before dilution and oblique column represents the inhibition after dilution. The urease activities before and after dilution were 36 and 24 μM NH_3/min per mg of protein, respectively.

3.4. Effect of preincubation time on the inhibition of urease activity

As shown in Fig. 4, ecabet gradually inhibited the enzyme activity with time and it took 20 min to reach the steady level of about 90% inhibition. In contrast, benzohydroxamic acid inhibited the enzyme activity within 3 min.

3.5. Reversibility of the urease activity

Reversibility of the inhibition of enzyme activity was determined by a dilution method (Dixon and Webb, 1979). Following preincubation for 10 min, urease activity was depressed by about 60% by ecabet at 4 mg/ml and benzohydroxamic acid at 1 μg/ml (Fig. 5). After 10-fold dilution, the inhibition of enzyme activity produced by benzohydroxamic acid was the same as that elicited by 0.1 μg/ml (data not shown). In contrast, there was no change in the ecabet-induced inhibition after the 10-fold dilution.

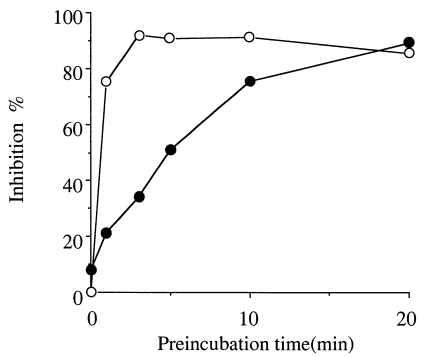

Fig. 4. Effect of preincubation time on the inhibition of urease activity by ecabet and benzohydroxamic acid. A crude enzyme preparation of *H. pylori* NCTC 11637 was added to the buffer containing the test drug, preincubated for fixed time at 37°C and then the activity was measured. Concentrations of ecabet (○) and benzohydroxamic acid (●) were 4 mg/ml and 10 μg/ml, respectively. Activities of control was 107 and 61 μM NH_3/min per mg of protein after preincubation of 0 (without preincubation) and 10 min, respectively.

4. Discussion

H. pylori is not viable at pH 3.0 unless urea is added in the medium. Since the urea concentration in the stomach is about 10 mM, which is higher than K_m value of the urease of *H. pylori*, the survival of *H. pylori* in gastric acid is likely to depend on the presence of urea in the stomach (Mobley et al., 1991; Marshall et al., 1990). Ecabet inhibits the urea-dependent survival of *H. pylori* in low pH media. In contrast, the anti-*H. pylori* action of ecabet is weaker as the pH increases (Shibata et al., 1995). Thus the ecabet-induced anti-*H. pylori* action is dependent on protons.

Ecabet has sulfonic and carboxylic groups. As the pK_a of the carboxylic group is 5.1, ecabet exists for 88%, 50%, and 0% in the nonionized form at pH 4.0, 5.0, and 8.0, respectively. Thus the molecular form but not the ionic form of the carboxylic group is related to the anti-*H. pylori* action of ecabet.

The urease activity of *H. pylori* was characterized by two pH optima, 4.5–5.0 and 8.0. At low pH, neutralization of the proton by the NH_3 produced is likely to play a crucial role in the acid resistance of *H. pylori* (Marshall et al., 1990). Another spiral bacterium, *H. muridarum*, which is nonviable at acidic pH in vitro as well as in stomach in vivo, contains urease with a high and low activity at neutral and acidic pH, respectively (Lee et al., 1993). Therefore, the urease activity at low pH may enable *H.*

pylori to survive in the gastric acid by neutralizing any hydrogen ion around the organism. In this respect, inhibition by ecabet of urease activity at pH 5.0 and lower seems to be related to its anti-*H. pylori* action. On short-term exposure, the lower the pH of medium, the greater the inhibition by ecabet of *H. pylori* urease activity. These results show that the anti-urease activity of ecabet is proton-dependent, similar to its anti-*H. pylori* action.

The ecabet-induced inhibition developed slowly and was not reversed by the dilution of ecabet. This is in contrast to the immediate inhibition of urease activity following incubation with benzohydroxamic acid, a specific and reversible urease inhibitor, and its complete reversal following dilution (Kobashi et al., 1962). These results indicate that ecabet irreversibly inhibits *H. pylori* urease activity. We have identified the characteristics of the anti-urease action of ecabet by using purified jack bean urease: ecabet irreversibly inhibits jack bean urease in a proton-dependent manner, and its mechanism is based on the denaturation of the enzyme (Ito et al., 1995). Similar to the proton-dependent irreversible inactivation of jack been urease, the inhibition of *H. pylori* urease by ecabet seems to occur through the denaturation of enzyme. Ecabet contains a carboxylic group and is present mainly in a molecular rather than an ionic form in proton-rich conditions. This form of ecabet easily binds and denatures the protein in low pH.

At acidic pH, ecabet inhibits peptic activity and enhances the protection provided by the gastroduodenal mucosa (Onoda et al., 1989; Ito et al., 1993a,b). The lack of absorption and activity of the ionic form at neutral pH in the intestine makes ecabet a locally acting agent in the stomach (Ito et al., 1991). Proton-dependent inhibition of urease activity is expected only in the stomach as well. Irreversible inhibition of the urease activity may be related to the anti-*H. pylori* action reported in *H. pylori*-infected monkeys (Fukuda et al., 1994, 1996) as well as patients with peptic ulcer (Fukuda et al., 1995; Isomoto et al., 1995).

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