Effects of the New Anti-Ulcer Drug Ecabet Sodium (TA-2711) on Pepsin Activity
I. Inactivation of Enzyme Protein

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ABSTRACT—To investigate the mechanism of the anti-peptic action of ecabet sodium (TA-2711) observed in pylorus-ligated rats, effects of this drug on the peptic activity of rat gastric juice, purified hog pepsin and pepsinogen were studied in vitro. After incubation with or without ecabet at acidic pH, the reaction mixture was centrifuged, and the peptic activity of the supernatant was measured. Ecabet depressed the peptic activity of pepsin and pepsinogen in parallel with a decrease in the protein concentration of the respective supernatant. Depression was greatest with pepsinogen (97% at 2.5 mg/ml of the drug) followed by gastric juice (about 60% at 10 mg/ml), and inhibition of the peptic activity of pepsin was weakest (about 10% at 10 mg/ml). When a fraction of the rat gastric juice containing substances with molecular weights below 10,000 was added to the pepsin solution, the anti-peptic activity of ecabet was potentiated. These results suggest that oral dosing of ecabet reduces the peptic activity of gastric juice by precipitating pepsin, which is facilitated by an unknown component(s) of gastric juice, and that the inactivation of pepsinogen may also contribute to the anti-peptic activity of ecabet.

Keywords: Ecabet (TA-2711), Anti-pepsin activity, Hog pepsinogen, Hog pepsin

It has been reported that ecabet sodium (ecabet, (+)-(1R,4aS,10aR)-1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-6-sulfo-1-phenanthrenecarboxylic acid 6-sodium salt pentahydrate, TA-2711, Fig. 1) prevents the development of various experimental ulcers in rats by acting locally in the upper gastrointestinal tracts (1–3). Its anti-ulcer action was suggested to involve both defensive and aggressive factors in the gastric mucosa: the drug enhances mucosal resistance and inhibits pepsin activity (1). The enhancement of mucosal resistance was considered to be mediated by an increase in mucosal prostaglandins (1, 3). It has been shown that oral dosing of ecabet reduced the peptic activity of gastric juice in pylorus-ligated rats (1). In the present study, we investigated the anti-peptic mechanism of ecabet by measuring the peptic activity of rat gastric juice, purified hog pepsin and pepsinogen after incubation with ecabet in vitro.

MATERIALS AND METHODS

Compounds and reagents
Ecabet was synthesized at the Organic Chemistry Research Laboratory, Tanabe Seiyaku. Bovine hemoglobin and purified hog pepsin (2,240 unit/mg) and pepsinogen (3,700 unit/mg) were purchased from Sigma (St. Louis, MO, USA). Folin’s reagent was obtained from Nacalai Tesque (Kyoto).
Preparation of rat gastric juice

Male Sprague-Dawley rats (Charles River Japan, Kanagawa; 7–8 weeks of age, about 180 g after fasting for 48 hr) were used. The animals were anesthetized with ether, and the pylorus was ligated according to the method of Shay et al. (4). Five hours after the ligation, the stomach was removed. The gastric contents from 5 animals were pooled and centrifuged at 1,000 × g for 10 min. The supernatant was used as gastric juice.

Effect of ecabet on peptic activity of gastric juice

Ecabet in various amounts was dissolved in the buffer at pH 1.6 (NaCl 2 g, 10% HCl 24 ml/l). After pre-incubation for 5 min of the solution (0.5 ml), an equal volume of gastric juice was added. The mixture was incubated at 37°C for 30 min, and then centrifuged at 1,000 × g for 10 min. A fifty-fold dilution of the supernatant was tested for peptic activity.

Effect of ecabet on pepsin and pepsinogen

Pepsin is unstable at higher pH, and pepsinogen is autocatalytically degraded at low pH (5). Therefore, purified hog pepsin was dissolved in the buffer at pH 1.6, and purified hog pepsinogen was dissolved in water. To allow ecabet to interact with the enzyme at acidic pH, after the ecabet solution (0.5 ml) was pre-incubated for 5 min, an equal volume of the pepsin solution or pepsinogen solution was added. The final concentration of pepsin and pepsinogen was 0.75 and 0.5 mg/ml, respectively. The mixture was incubated at 37°C for 30 min and then centrifuged. The supernatant was diluted to a 100-fold volume and then tested for peptic activity. The protein concentration of the supernatant was also determined.

In another series of experiments, purified hog pepsinogen was dissolved in the buffer at pH 1.6 and pre-incubated at 37°C for 15 min (acidified pepsinogen solution). The purpose of acidification was to obtain a pepsin solution containing the fragmental peptides derived from pepsinogen. The acidified or unacidified pepsinogen solution (0.1 ml) was added to 0.9 ml of ecabet solution: the final concentration of ecabet and pepsinogen was 5 mg/ml and 0.5 mg/ml, respectively. The subsequent procedure was the same as described above.

Effect of some components of gastric juice on anti-peptic activity of ecabet

Fractions of gastric juice containing substances with molecular weights below 10,000 and 30,000 were obtained by ultrafiltration of the gastric juice through Ultracento (Tosoh, Tokyo) and Centrifree MPS-3 (Amicon, Danvers, MA, USA), respectively. Each gastric juice fraction (0.2 ml) was mixed with the pepsin solution (0.1 ml) or pH 1.6 buffer (0.1 ml). Gastric juice (0.2 ml) was mixed with the buffer at pH 1.6 (0.1 ml). Each of these mixtures (0.3 ml) was then added to the ecabet solution (0.1 ml) and incubated at 37°C. Thirty minutes later, the mixture was centrifuged at 1,000 × g for 10 min, and a 100-fold dilution of the supernatant was tested for peptic activity. The final concentration of ecabet and pepsin was 5 mg/ml and 0.75 mg/ml, respectively.

Measurement of peptic activity

Peptic activity was determined by the method of Anson (6). The sample solution (0.2 ml) was taken into a tube, mixed with 1 ml of hemoglobin solution (dissolved in 0.06 N HCl, 10 mg/ml) and incubated at 37°C for 10 min. The reaction was terminated by adding 1 ml of 7% trichloracetic acid (TCA), and the mixture was centrifuged at 1,000 × g for 10 min. The supernatant (1 ml) was made alkaline by addition of 0.55 M Na2CO3 (5 ml), and then 1 N Folín’s reagent (0.5 ml) was added. After allowing the solution to stand for 30 min at room temperature, the optical density was measured at 660 nm by a spectrophotometer (UV-300, Shimadzu, Kyoto). The amount of TCA-soluble products was taken as the peptic activity and expressed as mg tyrosine/ml calculated from the standard curve for tyrosine.

Measurement of protein concentration of pepsin and pepsinogen solutions

Protein concentration was determined by the method of Lowry et al. (7). The solutions of 0.5% CuSO4 in 1% sodium succinate and 2% NaHCO3 in 0.1 N NaOH were mixed in a ratio of 1:50 (v/v) just before use. The resultant solution (5 ml) was added to the sample (0.1 or 0.05 ml) followed by addition of 1 N Folín’s reagent. After allowing the solution to stand for 30 min at room temperature, the optical density at 750 nm was read. The protein concentration was calculated from the standard curve for purified hog pepsin.

Statistical analysis

The correlation was calculated on the basis of least squares linear regression analysis. Statistical significance was determined by one-way analysis of variance followed by Bonferroni’s method. A P value of less than 0.05% was regarded to indicate a significant difference.

RESULTS

Effect of ecabet on peptic activity of rat gastric juice and solutions of pepsin and pepsinogen

The peptic activities of rat gastric juice and pepsin and pepsinogen solutions without ecabet were of almost the same level: 2.06±0.03, 2.22±0.13 and 2.13±0.05 mg
Kinetic analysis has shown that ecabet inhibits peptic hydrolysis as the result of a substrate-inhibitor interaction (8). In the present study, the samples containing ecabet were diluted 50 to 100-fold before measuring the peptic activity. We have observed no inhibition of peptic activity by ecabet at these diluted concentrations. Thus, the effect of the ecabet-substrate interaction should be negligible in this assay system.

After the incubation of gastric juice, pepsin or pepsinogen solution with ecabet, the peptic activity of each supernatant was decreased as the drug concentration increased (Fig. 2). The depression of peptic activity by ecabet was marked in the case of pepsinogen, being about 97% at the drug concentration of 2.5 mg/ml. On the other hand, ecabet at the highest concentration of 10 mg/ml inhibited the peptic activity of pepsin by about 10% and that of rat gastric juice by about 65%.

Following the incubation of pepsin or pepsinogen with ecabet, the protein concentration of each supernatant was decreased almost in parallel with the decrease in peptic activity (Fig. 3).

**Effect of ecabet on peptic activity of acidified pepsinogen solution**

Table 1 shows the effect of ecabet on the peptic activity and protein concentration of the supernatant after incubation of acidified and unacidified pepsinogen solutions.

In acidified and unacidified pepsinogen solution, the addition of ecabet resulted in the inhibition of peptic activity in the supernatant by 97% and 19%, respectively, which were parallel to the decrease in protein concentration.

**Effect of some component(s) of rat gastric juice on the inhibition of peptic activity by ecabet**

Table 2 shows the effect of a fraction of rat gastric juice activity by ecabet at these diluted concentrations. Thus, the effect of the ecabet-substrate interaction should be negligible in this assay system.

After the incubation of gastric juice, pepsin or pepsinogen solution with ecabet, the peptic activity of each supernatant was decreased as the drug concentration increased (Fig. 2). The depression of peptic activity by ecabet was marked in the case of pepsinogen, being about 97% at the drug concentration of 2.5 mg/ml. On the other hand, ecabet at the highest concentration of 10 mg/ml inhibited the peptic activity of pepsin by about 10% and that of rat gastric juice by about 65%.

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**Effect of some component(s) of rat gastric juice on the inhibition of peptic activity by ecabet**

Table 2 shows the effect of a fraction of rat gastric juice
containing substances with molecular weights below 10,000 on the peptic activity of pepsin after incubation with and without ecabet. The activity of pepsin was enhanced by the addition of this fraction; however, the mechanism for the enhancement is yet not clear. Incubation of the pepsin solution and gastric juice with ecabet reduced the peptic activity of the supernatant by 90% and 69%, respectively. Inhibition of the peptic activity of pepsin by ecabet was augmented in the presence of the above fraction of gastric juice (36%). A fraction containing substances with molecular weights below 30,000 also enhanced the inhibitory effect of ecabet to the same extent as the fraction of smaller molecular weights (data not shown).

The gastric juice component with molecular weights below 10,000 or 30,000 did not have any peptic activity.

**DISCUSSION**

It has been shown that instillation of HCl alone into the stomach and small intestine does not cause ulceration, but inclusion of gastric juice or pepsin with acid results in ulcer formation, suggesting that pepsin may be an important factor in ulcer formation (9, 10). The anti-peptic action of ecabet has been shown to be caused by a direct action on pepsin(ogen) secreted into the stomach (1), and this could be involved in its anti-ulcer effects (1–3). The results obtained in the present study explain more precisely the anti-peptic action of ecabet.

The depression of peptic activity in a pepsinogen or pepsin solution by ecabet correlated with the decrease in protein concentration in the supernatant (Fig. 3). This indicates that the peptic inhibition is caused by precipitation of pepsin or pepsinogen by ecabet. It seems that the precipitation is due to binding of ecabet to the enzyme proteins since ecabet was found to be bound to several kinds of protein including pepsin in a non-specific manner at acidic pH (8). It was also shown that the affinity of ecabet to pepsin was lower than that to albumin (8). Pepsin is poorer in basic amino acid residue contents than albumin or pepsinogen (5, 11, 12); thus, it may be difficult for the enzyme to attain a suitable conformation for ecabet binding. The difference in the binding ability of the drug to pepsin and pepsinogen might explain the difference in the inhibitory effect on peptic activity between these enzymes.

<table>
<thead>
<tr>
<th>Table 1. Effect of ecabet on peptic activity of acidified and unacidified pepsinogen solution</th>
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<td></td>
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<tr>
<td>Unacidified pepsinogen</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>2.18±0.15</td>
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<tr>
<td>Ecabet</td>
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<tr>
<td>0.07±0.03 (96)</td>
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<tr>
<td>Acidified pepsinogen</td>
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<tr>
<td>Control</td>
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<tr>
<td>2.05±0.05</td>
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<tr>
<td>Ecabet</td>
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<tr>
<td>1.66±0.04 (19)</td>
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After incubation of a mixture of ecabet (5 mg/ml, final) and pepsinogen or preincubated pepsinogen at acidic pH, the peptic activity and protein concentration of the supernatant were determined. The numbers in parentheses represent the % reduction of peptic activity and protein concentration. Each value represents the mean ± S.E. (n=4).

<table>
<thead>
<tr>
<th>Table 2. Effect of some component(s) of rat gastric juice on the inhibition of peptic activity of pepsin solution by ecabet</th>
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<td>gastric juice</td>
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<td>---------------------------------------------------------------</td>
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<td>Control</td>
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| Ecabet             | 0.73±0.06* (69) | 2.34±0.10 (9) | 1.93±0.05* (36)

A fraction of components with molecular weights less than 10,000 was obtained by ultrafiltration of the rat gastric juice. After the rat gastric juice or pepsin solution containing the components of which the concentration was almost equal to that of the gastric juice was incubated with ecabet (5 mg/ml, final), the peptic activity of the supernatant was determined. The numbers in parentheses represent the % reduction of peptic activity. Each value represents the mean ± S.E. (n=4). *P<0.05: significant difference from the peptic activity of each control. *P<0.05: significant difference from the peptic activity of the control of the pepsin solution.
The inhibition of peptic activity in the pepsin solution by ecabet was smaller than that in the gastric juice (Fig. 2). Inactivation of pepsinogen by ecabet did not seem to be involved in the inhibition in the gastric juice. In the gastric juice, which was collected for 5 hr in pylorus-ligated rats, pepsinogen could not be present since this proenzyme is rapidly converted to pepsin under acidic conditions (5, 13). The inhibition of pepsin was augmented by an unknown component(s) of rat gastric juice of which the concentration was almost equal to that of the gastric juice (Table 2), suggesting that the component was involved in the inhibition in the gastric juice. However, in the presence of the gastric juice components with molecular weight below 30,000, the inhibition was of the same level as that by the fraction below 10,000 (about 35%), and it was smaller than the inhibition in rat gastric juice (60–70%). Thus, involvement of some gastric juice components with molecular weights above 30,000 and/or some unknown factors cannot be excluded.

Some peptides which were derived from conversion of pepsinogen to pepsin might be the unknown gastric juice components mentioned above. However, in the acidified pepsinogen solution, the anti-peptic activity of the drug was much weaker than that in the unacidified pepsinogen (Table 1). Therefore, some other substances in the gastric juice seem to contribute to the reduction of peptic activity. Furthermore, these results also support that ecabet could be bound to pepsinogen directly to precipitate the proenzyme before conversion to pepsin.

We have observed that ecabet is distributed densely over the gastric mucosa after oral dosing (14, 15), which suggests that ecabet may interact directly with pepsinogen immediately after secretion into the gastric lumen. The present study showed that ecabet causes a much stronger inhibition of the peptic activity of pepsinogen than that of gastric juice or pepsin (Fig. 2). Therefore, precipitation of pepsinogen might possibly contribute to the inhibition of the peptic activity of rat gastric juice in vivo.

Several types of anti-pepsin agents, such as sucralfate, carbenoxolone and pepstatin, are known to act on the enzyme directly, and their mechanisms of action have been reported (16–19); sucralfate in its suspension form adsorbs pepsin at pH’s above 3 (16); carbenoxolone inhibits the peptic activity of purified pepsin by forming an insoluble complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); pepstatin is bound to an equimolar complex (17, 18); 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15 Ito, Y., Sugawara, Y., Takaiti, O. and Nakamura, S.: Metabolic fate of a new anti-ulcer drug (−)-(1R,4aS,10aR)-1,2,3,4, 4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-6-sulfo-1-phenanthrenecarboxylic acid 6-sodium salt pentahydrate (TA-2711). II. Distribution in the rat stomach. J. Pharmacobiodyn. 14, 547–554 (1991)


