Effects of the New Anti-Ulcer Drug Ecabet Sodium (TA-2711) on Pepsin Activity
II. Interaction with Substrate Protein

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ABSTRACT—To define the mechanism of the protection by ecabet (TA-2711) of the gastric mucosa from peptic attack, the characteristics of protein binding of this drug and its effect on peptic hydrolysis of substrate proteins were investigated in vitro. Both the binding to proteins and the hydrophobicity of ecabet were dependent on the pH; the lower the pH, the higher both parameters. The percentage of ecabet bound to proteins was nearly constant, being independent of the drug concentration at pH's below 2, indicating that this drug is bound to proteins in a non-specific manner. The activity of peptic hydrolysis of bovine serum albumin (BSA) decreased in the presence of ecabet, and this was not due to the interaction between pepsin and ecabet judging from the kinetic studies. The apparent Kₘ values of peptic hydrolysis of BSA increased depending on the quantity of ecabet bound to BSA. These results suggest that ecabet is bound to substrate proteins by a non-specific hydrophobic interaction to form a complex that is less vulnerable to peptic hydrolysis.

Keywords: Ecabet (TA-2711), Peptic hydrolysis, Protein binding

Ecabet sodium (TA-2711, ecabet, (+)-(R,4aS,10aR)-1,2, 3, 4, 4a, 9, 10, 10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-6-sulfo-1-phenanthrene carboxylic acid 6-sodium salt pentahydrate), a new anti-ulcer agent, possesses antipeptic activity that was shown to result from inactivation of pepsin and pepsinogen by precipitation of these enzymes (1, 2). We have observed that orally administered ecabet has a high affinity for the gastric mucosa, especially for necrotic areas, in rats (3, 4). This property seems to be involved in the protection of the gastric wall from peptic attack by gastric juice. The present study describes the characteristics of the protein binding of ecabet, which may be related to the binding of this drug to the gastric wall and its protective effect on proteins against peptic hydrolysis.

MATERIALS AND METHODS

Compounds and reagents

Ecabet was synthesized at the Organic Chemistry Research Laboratory, Tanabe Seiyaku. [¹⁴C]Ecabet was synthesized at Amersham International plc. (Buckinghamshire, UK). The radiochemical purity of [¹⁴C]ecabet was more than 99%, and its specific activity was 3.7 MBq/mg. Hog pepsin, bovine serum albumin (BSA), rat serum albumin (RSA), human serum albumin (HSA), hog gastric mucin and human plasma fibrinogen were purchased from Sigma (St. Louis, MO, USA). Folin's reagent was obtained from Nacalai Tesque (Kyoto) or E. Merck (Darmstadt, FRG).

Binding study

Binding of ecabet to serum albumin: The binding of ecabet was measured by the ultrafiltration method. BSA or RSA was dissolved in buffers at various pH's (0.1 N HCl – 0.1 M sodium citrate for pH 1.2, 3.0 and 4.0; 0.067 M phosphate buffer for pH 7.4). After pre-incubation of 1 ml of the protein solution at 37°C for 5 min, [¹⁴C]ecabet was added. Following further incubation at 37°C for 5 min to attain an equilibrium, the solution was ultra-
filtered at 600 × g for 10 min at 37 °C by using Centrifree MPS-3 (Amicon, Danvers, MA, USA). The concentration of radioactivity was measured before and after ultrafiltration; an aliquot of each sample was dissolved in scintillator (Hionic Fluor, Packard, Chicago, IL, USA) and measured by a liquid scintillation spectrophotometer (Packard Tri-Carb 460CD). The bound fraction was calculated by the following equation:

\[
\text{bound fraction (°Io)} = 100 - \left( \frac{\text{concentration after filtration}}{\text{concentration before filtration}} \right) \times 100
\]

**Binding of ecabet to HSA, mucin, fibrinogen and pepsin:** Binding of ecabet to several kinds of protein was determined by the above-described method. Following the incubation of the protein solution containing ecabet, the mixture was ultrafiltered. The concentration of ecabet was measured by HPLC. A Shimadzu LC-6A HPLC system (Kyoto) with a column of TSK gel 120T (Tosoh, Tokyo) was used. Acetonitrile/0.01 M potassium dihydrogen phosphate (25:75, v/v) was used as the mobile phase for isocratic elution. The eluate was monitored by ultraviolet (UV) absorption at 225 nm by a Shimadzu SPD-2A UV detector. The bound fraction was calculated by the equation described above. The buffers used to dissolve the proteins were as follows: HCl buffer at pH 1.6, glycine-HCl buffer at pH 2.0 and 3.0, acetate buffer at pH 4.0 and 5.0, and phosphate buffer at pH 6.7 and 7.5. The ionic strength of each solution was adjusted to 0.1 by adding KCl for the HCl buffer and NaCl for the others.

**Hydrophobicity of ecabet**

To determine the hydrophobicity of ecabet, partition coefficients of the drug in octanol/water (log P) were measured. [14C]Ecabet was dissolved in 3 ml of buffer at pH 1.2, 3.0, 4.9 or 7.4 at a final concentration of 0.5 mg/ml. The solution was mixed with 3 ml of octanol and centrifuged to separate the two phases. The radioactivities in the octanol and aqueous phases were measured by the liquid scintillation spectrophotometer.

**Peptic hydrolysis**

Peptic hydrolysis was measured with BSA as the substrate. BSA, ecabet or pepsin was dissolved in buffer at pH 1.2. Following the pre-incubation of the medium containing ecabet and BSA at 37 °C for 5 min, pepsin was added at the final concentration of 2 μg/ml. Two minutes later, 15% trichloroacetic acid (TCA, 1 ml) was added to the medium (3 ml) to terminate the reaction, and the mixture was centrifuged at 1,000 × g for 15 min. The supernatant (3 ml) and 1.5 M sodium carbonate (2 ml) was mixed, and 0.6 N Folin’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 with a spectrophotometer (Shimadzu, UV-300). The TCA-soluble products were expressed as μg tyrosine calculated from the standard curve for tyrosine.

**Digestion of gastric mucosa by artificial gastric juice**

The experiment was carried out according to the method of Daigo et al. (5). Male Sprague-Dawley rats (200–310 g after fasting, Charles River Japan, Tokyo) were starved overnight. The stomach was removed under ether anesthesia and reversed through the pylorus. The mucosal side of the reversed stomach was washed in physiological saline and each stomach was weighed. After ligation of the cardia and pylorus, the stomach was immersed in an artificial gastric juice (3 mg/ml pepsin and 2 mg/ml NaCl in 0.1 N HCl) containing ecabet or not. Following incubation at 37 °C for 30 min, 7% TCA (0.5 ml) was added to the medium (0.5 ml), and the mixture was centrifuged. The TCA-soluble products in the supernatant were determined as a parameter of the digestion of gastric mucosa in the same way as described above.

**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**

**Albumin binding of ecabet**

As shown in Table 1, the percentage of ecabet bound to BSA and RSA was higher at lower pH: about 90% at pH 1.2 and about 30% at pH 7.4. In this experiment the concentrations of ecabet and BSA were 0.3 mg/ml and 4 mg/ml, respectively.

Scatchard plots of the binding of ecabet (0.3–100

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**Table 1. Influence of pH on hydrophobicity (log P) and serum albumin binding of ecabet**

| pH | log P | Bound ecabet (%) |
|----|------|------------------|
|    |      | rat | bovine |
| 1.2 | 1.91 | 90.2 | 88.3 |
| 3.0 | 0.85 | 87.9 | 87.5 |
| 4.9 | 0.64 | 48.0 | 53.2 |
| 7.4 | −1.30 | 25.1 | 32.0 |

The hydrophobicity of ecabet was expressed as log P. P is the concentration ratio of ecabet in octanol/water. Protein binding was measured by the ultrafiltration method after incubation of rat or bovine serum albumin (4 mg/ml) with ecabet (0.3 mg/ml) at 37 °C. Each value represents the mean of two experiments.
Protection from Pepsin by Ecabet

pg/ml) to BSA (0.4 mg/ml) at several pH values are shown in Fig. 1. At pH 7.4, the specific binding of ecabet to BSA was observed with a $K_d$ value of 89.6 $\mu$M and with 6 moles of ecabet bound per mole of BSA. Both high and low affinity sites were observed at pH 4.9. At pH 3.0, non-specific binding, in addition to specific binding, was also observed. At pH 1.2, only non-specific binding was observed.

Binding of ecabet to HSA, mucin, fibrinogen and pepsin

As shown in Fig. 2, the percentage of ecabet bound to

\[
\frac{r}{C_f} = \frac{1}{K_d} + \frac{1}{C_f} \cdot \frac{1}{C_b}
\]

where $r$ is the molar ratio of bound ecabet/BSA, $C_f$ is the micromolar concentration of free ecabet, and $C_b$ is the micromolar concentration of bound ecabet. $K_d$ is the dissociation constant. Each point represents the mean of two measurements.

Fig. 1. Binding of ecabet to bovine serum albumin (BSA) at various pH's plotted according to the method of Scatchard. A mixture of ecabet (0.3-100 $\mu$g/ml) and BSA (0.4 mg/ml) was incubated at 37°C, and then the protein binding was measured by the ultrafiltration method. $r$: molar ratio of bound ecabet/BSA, $C_f$: micromolar concentration of free ecabet. Molecular weights of ecabet and BSA are 493 and 69,000 (6), respectively. Each point represents the mean of two measurements.

Fig. 2. Influence of pH on the binding of ecabet to proteins. The binding study was carried out by the ultrafiltration method. Concentrations of ecabet and proteins were 0.2 and 8 mg/ml, respectively. ○: human serum albumin, □: fibrinogen, ▲: mucin, △: pepsin. Each value represents the mean of two measurements.

Fig. 3. Binding of ecabet to various proteins under the acidic condition. Proteins (8 mg/ml) were incubated with ecabet (0.0125-0.4 mg/ml) in buffer at pH 1.6 at 37°C. Protein binding was measured by the ultrafiltration method. ○: human serum albumin, ●: fibrinogen, ▲: mucin, □: pepsin. Each value represents the mean of two measurements.
the proteins, especially to HSA and fibrinogen, became higher as the pH was lowered.

At pH 1.6, the highest percentage of bound ecabet was observed with HSA, followed by fibrinogen, mucin and pepsin in this order (Fig. 3). The percentages of bound ecabet in the HSA, fibrinogen, mucin and pepsin solutions were nearly constant, independent of the ecabet concentration.

Hydrophobicity of ecabet

The partition coefficients of ecabet in octanol/water (log P) are shown as an index of hydrophobicity in Table 1. Figure 4 shows the relationship between pH and P or the percentage of non-ionic forms of the carboxyl (pKa = 4.8) and sulfonic (pKa = 2.7) groups calculated theoretically. As the pH decreased, P became larger, with an abrupt change between pH 3.0 and 1.2, which virtually corresponded to the percentage of non-ionized sulfonic groups (Fig. 4).

Effect of ecabet on peptic hydrolysis

Tyrosine content in the medium containing BSA and pepsin increased linearly with incubation time up to 2 min. Peptic hydrolysis was expressed as v (the rate of the tyrosine production).

Ecabet at 50–125 μg/ml inhibited the peptic hydrolysis of BSA (1.45–7.25 μM) in a concentration-dependent manner: 50–125 μg/ml of the drug caused 59.9–95.0% inhibitions of peptic hydrolysis of 1.45 μM of BSA. Figure 5 shows the effect of ecabet on peptic hydrolysis using Lineweaver-Burk plots. The K_m and V_max values of peptic hydrolysis were 2.67 μM and 1.07 μg tyrosine/ml/min, respectively. The plot revealed no linearity of 1/v as a function of 1/S (S: substrate concentration) in the presence of ecabet. V_max was constant, being independent of the concentration of the drug.

The equation $K_m = S \times \left( \frac{V_{max}}{v} - 1 \right)$ was derived from the Michaelis-Menten equation. The apparent K_m values were calculated by substituting the values of V_max and v obtained from the experiments in this equation. The molar ratios of bound ecabet/BSA were calculated according to the Scatchard analysis of the binding of ecabet to BSA in Fig. 1. Figure 6 shows the relationship between the K_m...
and the molar ratio of bound ecabet/BSA. The log $K_m$ value increased in proportion to the amount of ecabet bound to BSA.

**Effect of ecabet on gastric mucosal digestion**

Digestion of rat gastric mucosa induced by the artificial gastric juice was inhibited by the addition of 2–8 mg/ml of ecabet (Table 2). Little change in the pH of the artificial gastric juice was observed by the addition of ecabet.

**DISCUSSION**

In the present study, it was observed that the binding of ecabet to BSA was dependent on pH of the buffer solutions (Table 1). Such a pH dependency has been also reported in the binding of sucrose sulfate to BSA or fibrinogen, which is thought to be due to an ionic interaction between the negative charge of the dissociated sulfate moiety of the drug and the positive charge of proteins (7, 8). Thus, a similar mechanism can be assumed in the binding of ecabet to BSA. However, the ionic interaction would not be involved in the ecabet binding at low pH since the percentage of its ionized form was less than 4% at pH 1.2 (Fig. 4). Other types of drug-protein binding forces are dipole-dipole interaction and van der Waals-London forces (9), which are thought to result from hydrophobic interactions.

The hydrophobicity (P value) of ecabet was also shown to be pH-dependent (Table 1), and it was related to the percentage of the ionized sulfonic group (Fig. 4). This could be explained as follows: the P value of an ionic compound can be proportional to the percentage of its undissociated form, which was derived from Butler’s equation (10) and the Henderson-Hasselbalch equation, and so the percentage of the undissociated form of ecabet is mainly dependent on that of the non-ionized sulfonic group of which the pKa is lower than that of the carboxyl group (Fig. 4). At pH 7.4, the hydrophobicity of ecabet is low since its log P at that pH was −1.30 (Table 1), which is almost the same as that of water (11). On the other hand, ecabet has a high hydrophobicity at pH 1.2 as its log P was 1.91 (Table 1). Thus, the hydrophobicity of ecabet seems to contribute to the binding force between this drug and BSA at pH 1.2. However, at higher pH, the binding force could not be explained only by the hydrophobicity of ecabet since the pH-dependency of the ecabet binding was not parallel to that of the hydrophobicity (Table 1). Some other interactions, such as an ionic one, may also be involved in the binding of ecabet to BSA at higher pH.

Analysis of the binding by the method of Scatchard (Fig. 1) indicates that the binding of ecabet to BSA was non-specific at pH’s lower than 3. In addition, ecabet seems to be bound to several kinds of protein in a non-specific manner at low pH because the percentage of ecabet bound to each proteins at pH 1.6 was nearly constant regardless of the ecabet concentration (Fig. 3). These results suggest that ecabet is bound to proteins by non-specific hydrophobic interactions at low pH.

The pattern of the Lineweaver-Burk plot for the peptic hydrolysis (Fig. 5) indicates that the inhibition by ecabet would be caused by a substrate-inhibitor interaction (7, 8, 12–15). In addition, the apparent $K_m$ values of the peptic hydrolysis against the BSA-ecabet complex, which reflects the affinity of the enzyme for the complex, increased depending on the amount of ecabet bound to BSA (Fig. 6). Thus, the binding of ecabet to BSA seems...
to protect the substrate from pepsin. Ecabet was also bound to other proteins in non-specific manners as mentioned above. It seems that ecabet would inhibit the pepsic hydrolysis by binding to various substrates including the gastric mucosa. The protection of the gastric mucosa from pepsic hydrolysis by this drug was shown in the present study (Table 2).

The previous study has suggested that ecabet exerts anti-peptic action by precipitating pepsin (2). On the other hand, the present kinetic study indicates that the peptic inhibition by ecabet might also be caused by a substrate-inhibitor interaction. This divergence could be explained by the difference of ecabet concentration between these studies; the precipitation of pepsin (2) requires higher ecabet concentrations than those needed for the substrate protection. The affinity of ecabet to the substrate may be much higher than that to pepsin.

Inactivation of pepsin could be related to the inhibition of gastric mucosal digestion as mentioned above since the concentration of ecabet was 2-8 mg/ml, which was higher than that in the other experiments of the present study. However, the effect on purified pepsin has been shown to be small, being less than 10% (2). In this study, about 50% inhibition was observed. Therefore, substrate protection would be mainly involved in the inhibition of gastric mucosal digestion.

We have observed that orally administered ecabet was densely distributed over the gastric mucosa (3, 4). These observations can be partly explained by the binding of ecabet to the mucus layer. The gastric mucosal surface is covered with the mucus gel, which is formed by mucin at the concentration of 50-100 mg/ml (16, 17). The concentration of mucin in the mucus is much higher than that used in the present study (8 mg/ml). Generally, as the protein concentration increased, the amount of compound bound to the proteins becomes higher. Thus, it is considered that ecabet is bound to the gastric mucus layer in large quantities, despite of relatively low binding ability to mucin under these experimental conditions. Furthermore, it has been shown that ecabet is more heavily distributed to the ulcerous areas than to the intact mucosa (4). This property could account for the binding of this drug to serum and mucus proteins exuded from necrotic tissues (18) since the binding ability of this drug to these proteins was high (Fig. 3). The binding to mucus and the mucosal surface would result in fortification of the mucus-mucosal barrier, which is one of the defensive factors (19), and the binding to the ulcer site would result in the promotion of ulcer healing (20) through the protection of ulcer sites from the peptic attack by gastric juice.

In conclusion, it is suggested that ecabet is bound to proteins by a non-specific hydrophobic interaction to form a complex resistant to peptic hydrolysis, which would contribute to its anti-ulcer effect.

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