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Research Article

Ecabet sodium prevents esophageal lesions induced by the reflux of gastric juice in rats

K. Okuyama*, N. Saito, E. Kume, T. Noto and M. Nagasaki

Tanabe Seiyaku Co., Ltd. Pharmacology Research Laboratories, 2-2-50 Kawagishi, Toda Saitama 335-8505, Japan, Fax: ++81-48-433-8161, e-mail: kay@tanabe.co.jp

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Abstract. We investigated the effect of ecabet sodium (ecabet) on rat acute esophageal lesions induced by reflux of gastric juice. Ligation of both pylorus and fore-stomach induced the reflux of gastric juice, decreased the amount of mucus and formed hemorrhagic lesions in the esophageal mucosa. Intragastric injection of ecabet reduced the pepsin activity and prevented both the decrease of mucus amount and formation of lesions. Ecabet enhanced the reduction in lesion formation induced by omeprazole and ranitidine without a change in decreased acid concentration and pepsin activity. Digestion of mucosa and the reduction in mucus were prevented by ecabet in the everted HCl and pepsin treated esophageal sac. These results indicate that ecabet prevents esophageal lesions by inhibiting pepsin activity as well as by protecting mucus from degradation. These further implicate the therapeutic potential of ecabet for prevention/treatment of GERD, especially in combination with a proton pump inhibitor or H_2-antagonist.

Key words: Reflux of gastric juice; Esophageal lesion; Esophageal mucus; Ecabet sodium

Introduction

Gastro-esophageal reflex disease (GERD) is a clinical condition that occurs when reflux of gastric acid into the esophagus is severe enough to damage the esophagus. When accompanied with esophageal erosion or ulcer, inhibitors of acid secretion including proton-pump inhibitors and H_2-receptor antagonists will treat patients with GERD (Castell et al., 2002). Patients with a normal esophagus upon endoscopy are described as having non-erosive reflux disease (NERD). Only 60 to 70 % of patients with NERD respond to a treatment with suppressants of gastric secretion (Armstrong et al., 2004). While about 50 to 60 % of patients with systemic sclerosis are accompanied with GERD, these patients are often refractory to the treatment with gastric acid suppressors (Inn et al., 2002).

Ecabet sodium (ecabet), a gastric protectant, inhibits pepsin activity (Ito et al., 1993a), protects the gastric mucosal layer in various types of stress conditions (Ito et al., 1993b; Kinoshita et al., 1999a) and thus is used for the treatment of gastiritis and gastric ulcers. In patients with systemic sclerosis complicated by GERD, adjunct therapy with ecabet on the standard treatment improved the therapeutic efficacy to more than 90 % (Inn et al., 2002). A previous study has shown alleviation of gastric reflux-induced esophageal lesion by ecabet in rats (Omura et al., 2000). In the present study we have elucidated the detailed mechanism of this protection.

Methods

1. Animals and materials

Male Sprague-Dawley rats were purchased from Charles-River Japan at 6 weeks of age, kept in controlled conditions with room temperature of 23 ± 1 °C, relative humidity of 55 ± 5 % and 12-h light/dark cycles (light from 7:00 a.m. to 7:00 p.m.) and freely accessible to water and chow for 1 week. The animals (190–250 g) were fasted for 24 h before the experiment. Ecabet, omeprazole, ranitidine and teprenone were synthesized and rebamipide was extracted in Tanabe Seiyaku. For in vivo studies, the compounds were prepared in 0.25 % carboxymethyl cellulose sodium salt to administer as 1 mL/kg and 2 mL/kg for intragastric and intraduodenal injections, respectively. For in vitro studies, ecabet sodium was dissolved in 0.2 % NaCl containing 0.1 N HCl and 1 mg/mL pepsin (Sigma No P6887, 3640 units/mg protein).

2. Effects on esophageal lesions induced by reflux of gastric juice

The reflux of gastric juice was induced according to the method described previously (Inatomi et al., 1991). Under anesthesia with ether, the abdomen was opened and pylorus and the border of fore-stomach and

* *Corresponding author*

glandular stomach were ligated. Either ecabet or vehicle was injected into the stomach and the abdomen was sutured.

Rats were euthanized under deep anesthesia with ether 4 h after surgery and the 5 cm long esophagus and the whole stomach were removed. The esophagus was cut along with the longitudinal axis and the length of the hemorrhagic erosion was measured. Then the tissues were stored frozen at –20 °C and used for the determination of mucosalbound ecabet by HPLC. Gastric contents were centrifuged at 1,500 × g for 10 min and the acid concentration and pepsin activity in supernatant, i. e. gastric juice, were determined. Acid concentration was determined by a titrator (TTT2 Titrator, Radiometer) and expressed as μEq/mL. The pepsin activity was determined with hemoglobin as a substrate and was expressed as mg tyrosine/mL/10 min (Anson, 1938).

The content of mucus was determined as the amount of alcian blue bound on the mucosal layer (Corne et al., 1974). One and 2 h after the ligation, the esophagus was removed, gently everted without damaging the mucosal layer, washed with ice-cold saline and immersed in 0.16 M sucrose-0.05 M Na acetate buffer (pH = 5.8) containing 0.1 % alcian blue for 2 h in room temperature. Then the tissue was rinsed in 0.25 M sucrose and sonicated in 0.5 M $MgCl_2$ for 30 s. The alcian blue concentration in the solution was determined at 579 nm by a spectrophotometer. The esophagus of some animals were dissected 4 h after the ligation, fixed in buffered 10 % formalin, dehydrated and embedded in paraffin. Thin sections were stained with hematoxyline-eosine and examined under a light microscope.

Ecabet was administered directly into the stomach at 3, 10 and 20 mg/kg. Omeprazole at 3 and 10 mg/kg, ranitidine, rabamipide and teprenone at 100 mg/kg, respectively, were given into the duodenum.

3. Effect on digestion of mucus in isolated and everted esophagus sac

Rats were euthanized under deep anesthesia with ether and the esophagus of 5 cm long was isolated. Connective tissues were removed and the wet weight was measured. The esophagus was then everted, ligated with the both ends and digested in 0.2 % NaCl containing 0.1 N HCl and 1 mg/mL pepsin at 37 °C for 30 min. Ecabet was included in the solution at concentrations of 0.3, 1 and 3 mg/mL. Some tissues were incubated in 0.2 % NaCl and served as non-digested tissues. After the digestion, the esophagus was rinsed in ice-cold saline and stored in –20 °C. The amount of bound ecabet was determined by an HPLC method. The incubated solution was treated with the same volume of 7 % trichloroacetic acid and centrifuged at 1,500 × g for 10 min. The peptide content in supernatant was determined with tyrosine as a standard (Folin and Ciocalteau, 1927). The values under the detection limit were treated as 0 in the calculation. In other tissues, the mucus content was determined as the amount of bound alcian blue.

4. Analysis

Results were expressed as the means ±S.E.M. Statistical analysis was made by a *t*-test for comparison of 2 groups and one way analysis of variance followed by the Bonferroni's method for multiple groups. P values <0.05 were considered as statistically significant.

Results

1. Effects on esophageal lesions induced by reflux of gastric juice

Ligation of both pylorus and the border between the glandular- and fore-stomach formed hemorrhagic lesions in the esophageal mucosa within 4 hrs and the length of the erosions was 38.1 ± 5.4 mm. Ecabet dose-dependently suppressed the formation of the erosions at 3, 10 and 30 mg/kg (Fig. 1). There was a statistical significance at doses over 10 mg/kg. While the acid concentration was not affected, the pepsin activity was decreased and there was a binding of ecabet on mucosal layers. The pathological examination revealed extensive hemorrhages and necrosis not only in the mucosal epithelium but also submucosa and muscle layers in control animals 4 h after the ligation (Fig. 2). In tissues treated with ecabet, there was only a slight thinning of the cornified layer and reduced staining.

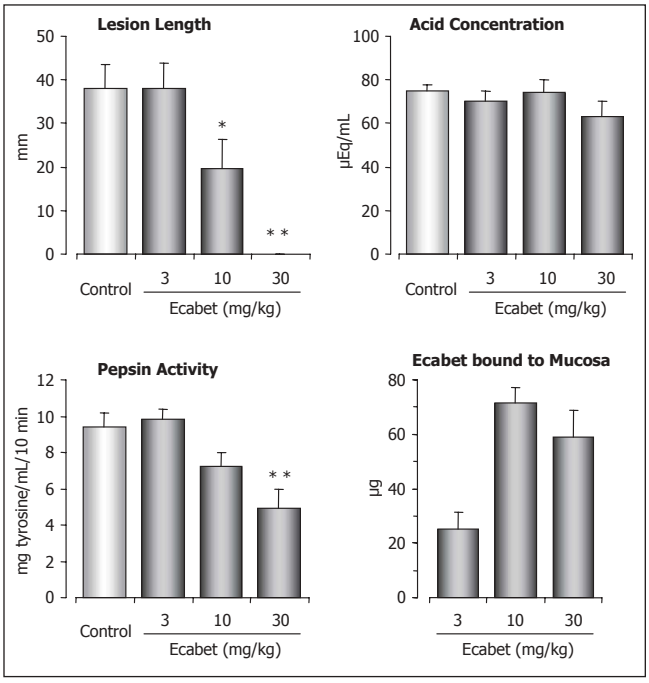

Fig. 1. Effects of intragastric ecabet on the esophageal lesions induced by gastric reflux.
Under anesthesia with ether, fore-stomach and pylorus were ligated. Four hours after the ligation, the stomach and esophagus were excised. The effects of ecabet on the length of hemorrhagic erosions, acid concentration and pepsin activity in the gastric juice and the amount of bound ecabet on the mucosal layer are shown. Data are the mean ± S.E.M of 7 to 9 animals. Significantly different from control *: p <0.05, **: P <0.01.

Omeprazole (10 mg/kg) and ranitidine (100 mg/kg) significantly reduced the acid concentration and the formation of mucosal lesions (Fig. 3). Rebamipide and teprenone both at 100 mg/kg neither affected the acid concentration and pepsin activity and nor inhibited the lesion formation.

2. Effects on the factors affecting the formation of mucosal lesions

Figure 4 illustrates the time course of the formation of mucosal lesion, acid concentration and pepsin activity in the gastric juice and the amount of bound ecabet on the mucosal layer following the ligation. Hemorrhagic lesions appeared 3 h after the ligation and are formed in all the animals at 4 h. Both omeprazole at 10 mg/kg and ecabet at 20 mg/kg significantly reduced the formation of mucosal lesions, acid

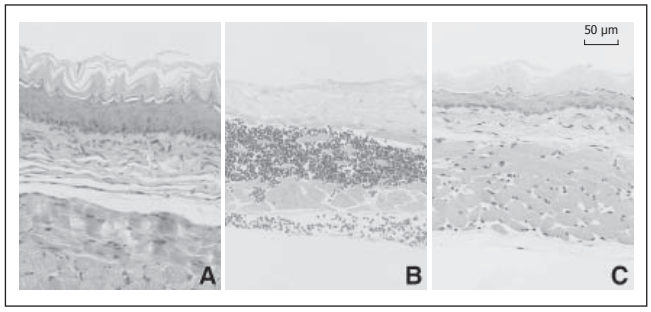

Fig. 2. Pathological changes of the esophagus following ligation of stomach.
Under anesthesia with ether, the pylorus and fore-stomach were ligated. Four hours after the ligation, the esophagus was dissected and fixed. The paraffin sections were stained with hematoxyline and eosine. A: normal, B: control, C: ecabet(20 mg/kg)-treated.

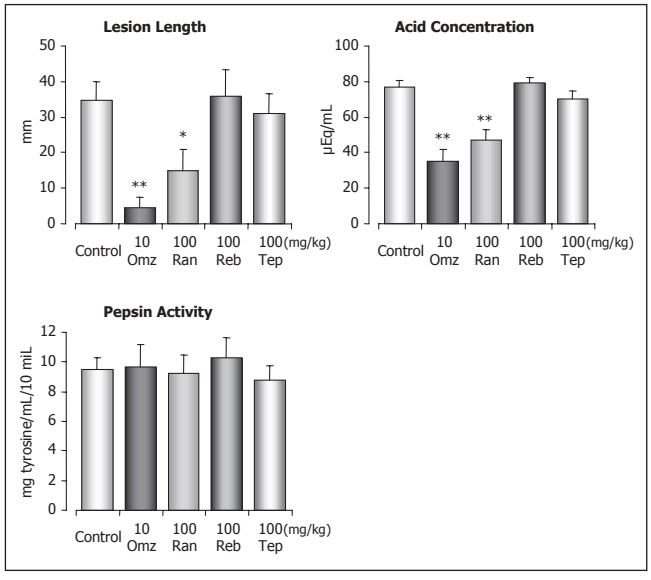

Fig. 3. Effects of omeprazole, ranitidine, rebamipide and teprenone on esophageal lesions induced by gastric reflux.
Under anesthesia with ether, fore-stomach and pylorus were ligated. Omeprazole (Omz 10 mg/kg), ranitidine (Ran, 100 mg/kg), rebamipide (Reb, 100 mg/kg) and teprenone (Tep, 100 mg/kg) were administered into duodenum. Four hrs later both the stomach and esophagus were dissected. The length of hemorrhagic lesions, acid concentration and pepsin activity in gastric juice were determined. Data are expressed as the mean ± S.E.M of 8 to 9 animals. Significantly different from control *: p <0.05, **: P <0.01

concentration and pepsin activity in 4 h time. Mucosal binding of ecabet increased upon time.

Figure 5 shows the esophageal mucus contents 1 and 2 h following the ligation. The mucus contents significantly reduced in 1 h and decreased to 18 % of the normal level in 2 h. Because of the formation of hemorrhagic lesions, the binding of alcian blue was not determined after 3 h. Both omeprazole and ecabet significantly prevented the reduction amount of mucus.

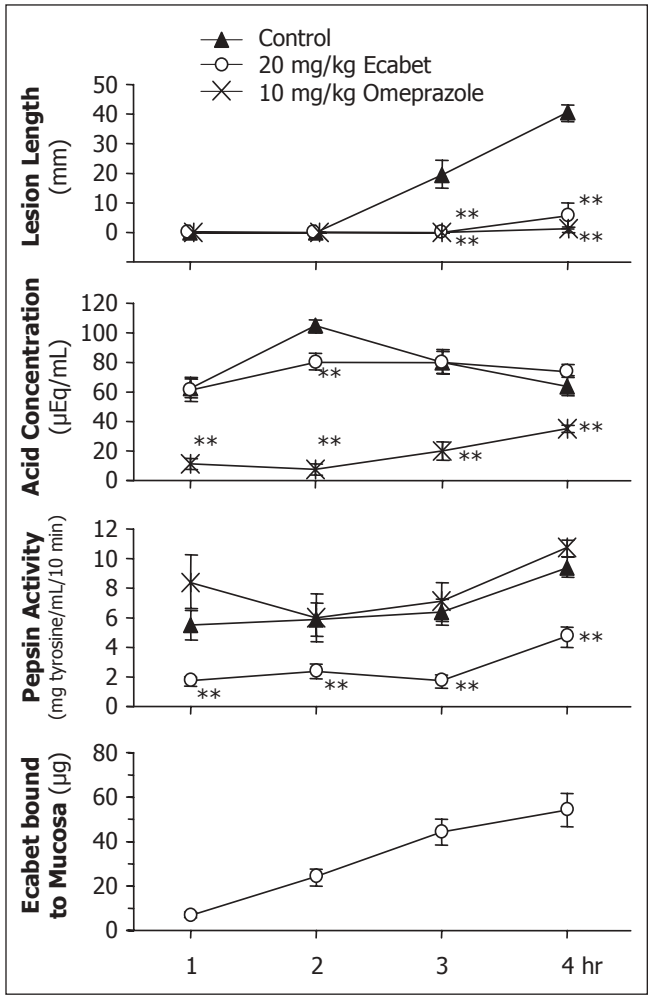

Fig. 4. Time course of effects of ecabet and omeprazole on the formation of esophageal lesions.
Under anesthesia with ether, fore-stomach and pylorus were ligated. Both the stomach and esophagus were excised 1, 2, 3 and 4 hrs after the ligation. Effects of ecabet (20 mg/kg i.g.) and omeprazole (10 mg/kg i.d.) were determined on the length of hemorrhagic erosions, acid concentration and pepsin activity in the gastric juice. The amount of bound ecabet on the mucosal layer was also examined. Data are the mean ± S.E.M of 7 to 10 animals. Significantly different from control *: p <0.05, **: P <0.01

3. Effect of ecabet on the digestion of isolated esophagus by pepsin and HCl

Artificial gastric juice containing pepsin and HCl digested mucosal layer of the isolated and everted esophagus sac were incubated. The amount of released peptide in the incubating solution increased to 734 ± 72.4 μg tyrosine/g wet weight (Fig. 6) and the content of remaining mucus decreased to 33 % by incubation with acid and pepsin. Ecabet dose-dependently inhibited both the release of peptide and the remaining mucus content on the everted sac at doses over 0.3 and 1 mg/mL, respectively. In parallel to the inhibition of digestion by artificial gastric juice, the amount of ecabet bound on the mucosa was increased dose-dependently.

Table 1. Effect of ecabet alone and in combination with omeprazole and ranitidine on gastric reflux-induced esophageal lesions

Treatment	Lesion Length mm	Acid Concentration μEq/mL	Pepsin Activity mg tyrosine/mL/10 min	Ecabet bound to Mucosa μg
Control	44.2 ± 2.8	59.3 ± 3.0	8.3 ± 0.7	
Ecabet	26.5 ± 4.3[a]	55.4 ± 4.7	6.1 ± 0.5	58.7 ± 13.3
Omeprazole	25.5 ± 3.9[a]	48.6 ± 3.6	10.1 ± 0.6	
Ranitidine	27.0 ± 3.0[a]	39.5 ± 4.2[a]	8.1 ± 0.6	
Ecabet + Omeprazole	6.4 ± 3.0[a,b,c]	44.6 ± 4.5	7.2 ± 0.6	38.8 ± 5.8
Ecabet + Ranitidine	6.1 ± 2.8[a,b,d]	40.7 ± 4.1[a]	6.7 ± 0.7	31.8 ± 6.0

Under anesthesia with ether, fore-stomach and pylorus were ligated. Ecabet (10 mg/kg) was administered into the stomach and omeprazole (10 mg/kg) and ranitidine (100 mg/kg) were into the duodenum. Four hours later, both the stomach and esophagus were dissected. The length of hemorrhagic lesions, acid concentration and pepsin activity in gastric juice and the amount of ecabet bound on the mucosal layer were determined. Data are expressed as the mean ± S.E.M of 13 to 16 animals. Significantly different (P <0.01) from control[a)], ecabet[b)], omeprazole[c)] and ranitidine[d)].

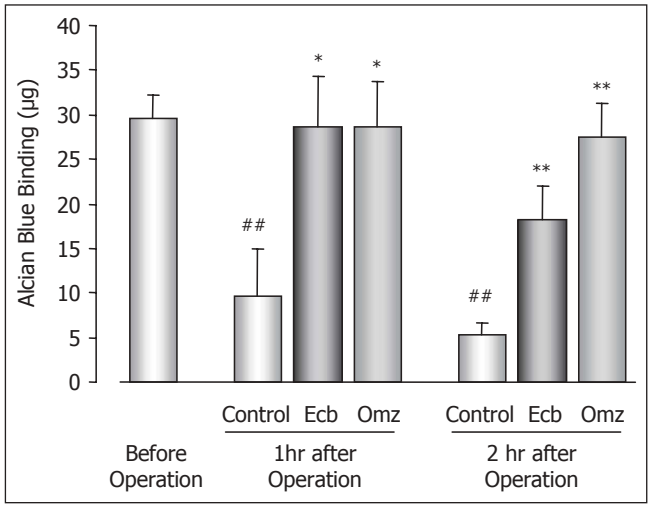

Fig. 5. Effect of ecabet and omeprazole on the mucus content of the esophagus following gastric reflux.
Under anesthesia with ether, fore-stomach and pylorus were ligated. Both the stomach and esophagus were excised 1 and 2 hrs after the ligation. Effects of ecabet (20 mg/kg i.g.) and omeprazole (10 mg/kg i.d.) were determined on the binding of alcian blue. Data are the mean ± S.E.M of 8 to 9 animals. Significantly different from control *: p <0.05, **: P <0.01 and from before operation ##: p <0.01

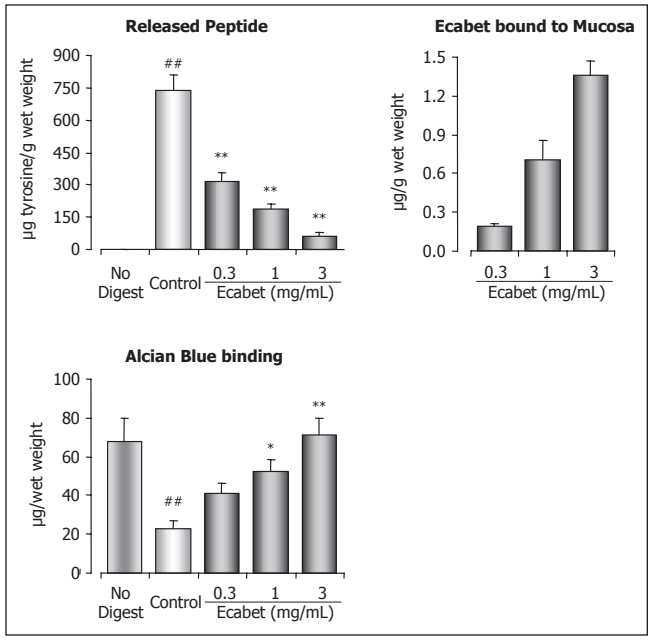

Fig. 6. Effect of ecabet on digestion of isolated esophagus by HCl and pepsin.
Isolated and everted esophagus sac was digested in 0.2 % NaCl containing 0.1 N HCl and 1 mg/kg pepsin at 37 ˚C for 30 min. No digest group was treated with 0.2 % NaCl. Data are the mean ± S.E.M of 6 to 8 animals. Significantly different from control *: p <0.05, **: P <0.01. and from no digest ## p <0.01.

4. Combination treatment of ecabet with acid suppressants

Table 1 shows the effect of ecabet in combination with omeprazole or ranitidine on esophageal lesions induced by ligation. Hemorrhagic lesions, 40 mm or more in control animals, were reduced by about 40 % with either treatment alone. Combined treatment of ecabet with omeprazole or ranitidine enhanced the inhibitory effect of each treatment. The formation of erosion was reduced to about 6 mm or by 85 % of the control level. The suppression of pepsin activity by ecabet was not changed while the amount of bound ecabet to mucosa was tended to decrease by omeprazole and ranitidine. Ecabet did not affect the reduction of acid concentration by omeprazole and ranitidine.

Discussion

Ligation of pylorus and fore-stomach induces reflux of gastric juice into the esophagus and thus forms lesions in the esophageal mucosa in rats. In the present study, hemorrhagic and necrotic lesions were extensively induced within 4 h following the ligation in all animals. The mucus contents were profoundly reduced with no hemorrhagic lesions 2 h after the ligation. This indicates that the mucus reduction precedes the formation of erosion. A previous study has reported that the removal of salivary glands extensively reduces mucus contents and aggravates reflux-induced lesion forma-

tion (Kinoshita et al., 1999b). Therefore sufficient supply of mucus seems to be essential in preventing the esophageal lesions by reflux of gastric juice.

Ecabet counteracts the digestion of mucus contents by not only inhibiting the pepsin activity (Ito et al., 1993a) but also by forming a pepsin-resistant complex with albumin, fibrinogen and mucin (Ito et al., 1993b) and this mechanism underlies the protection of the mucosal layer and epithelium in the stomach. In the present study, treatment with ecabet reduced both the pepsin activity in gastric juice and the decrease of mucus content from the early phase of lesion formation following acute gastric reflux. The effect was maintained up to 4 h and the formation of hemorrhagic erosion was substantially inhibited in the esophagus. Digestion of esophageal mucus by artificial gastric juice in vitro was also inhibited by ecabet depending on the amount bound to the mucosa. The results thus indicate that ecabet preserves the integrity of the mucosal layer in esophagus by an effect on mucus contents as well as pepsin activity. The binding of ecabet to mucus may contribute to the protection of the mucosal layer. The mechanism distinct from inhibitors of gastric secretion confirms the potential of ecabet as a novel therapeutic for GERD.

Omeprazole and ranitidine, both inhibitors of gastric secretion, reduced acid concentration without effect on pepsin activity, and inhibited the hemorrhagic lesions in the esophagus induced by ligation of the stomach. Suppression of acid secretion explains well the clinical efficacy of acid suppressants in the treatment of GERD. In patients with refractory GERD, more than 90 % recovery was reported by ecabet in combination with the standard treatment (Inn et al., 2002). In the present study, ecabet significantly enhanced the inhibitory effect of omeprazole and ranitidine on ligation-induced lesion formation. Inhibition of pepsin activity by ecabet and suppression of gastric secretion by omeprazole and ranitidine did not affect each other. While gastric acid alone digests esophageal mucosa, the combination of pepsin and acid aggravates the lesion formation (Goldberg et al., 1969; Lillemoe et al., 1982; Lanas et al., 1997). Thus the enhanced protection by combination of ecabet and inhibitors of gastric secretion may be explained by additive coordinated action on pepsin activity and acid secretion. This implies that ecabet has potential as an adjunct for treatment of patients with GERD, especially refractory to inhibitors of acid secretion.

In conclusion, ecabet dose-dependently inhibited the formation of esophageal lesions induced by reflux of gastric juice in rats. Both inhibition of pepsin activity and protection of mucus from digestion by direct binding contribute to preservation of the mucosal layer. Combination of ecabet with an inhibitor of gastric secretion, i. e. omeprazole and ranitidine, further enhanced the inhibition of lesion formation. This demonstrates the potential of ecabet as a novel therapeutic for GERD and as an adjunct to the treatment of refractory GERD.

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