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Anti-Ulcer Activity of Newly Synthesized Acylquinoline Derivatives

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Anti-ulcer activity of newly synthesized acylquinoline derivatives was investigated. For the *in vitro* screening, the effects of the compounds on gastric H^+/K^+ ATPase isolated from hog and rabbit were examined. Among them, AU-090, AU-091, AU-254, AU-413 and AU-466 exhibited good *in vitro* activity on both enzymes. To correlate the *in vitro* activity with *in vivo* action, the effects of the compounds on the basal gastric acid secretion were studied. Some derivatives showed considerable anti-secretory activities, and AU-413 was selected for further studies. AU-413 protected gastric damage induced by either ethanol or NaOH dose dependently when given orally. ED_{50} values of 12 mg/kg, *p.o.* (ethanol) and 41 mg/kg, *p.o.* (NaOH) were obtained. In addition, histamine-stimulated gastric secretion was reduced upon AU-413 administration. Taken together, newly synthesized acylquinoline derivatives, especially AU-413, is worthy of further investigation to be developed as an anti-ulcer agent.

Key words : H^+/K^+ ATPase, Gastric acid, Anti-ulcer agent

INTRODUCTION

The proton pump, located in gastric parietal cells has been recently targeted for peptic ulcer therapy (Sachs *et al.*, 1976). Since it is involved in the final process of gastric acid secretion, the blockade of the proton pump is expected to be a selective and effective way in reducing gastric acid secretion. In addition, proton pump is known to have H^+/K^+-dependent ATPase activity (Ganser and Forte, 1973). From the characterization of the genes cloned from rats and humans, it was revealed that gastric H^+/K^+ ATPase was composed of α (114 kDa) and β (33 kDa) subunits, α subunit having the catalytic activity (Shull, 1990; Maeda *et al.*, 1990).

Substituted benzimidazole derivatives have been shown to be irreversible proton pump inhibitors (Gustavsson *et al.*, 1983). Omeprazole clinically used at present is included as a prototype of substituted benzimidazole derivatives. In our laboratory, various acylquinoline derivatives were designed, synthesized and tested for the anti-ulcer activity to evaluate their possible use as new anti-ulcer agents. Using various pharmacological screening methods, the anti-ulcer activity of selected compounds was examined, and AU-413 appears to be a suitable candidate for further investigation.

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MATERIALS AND METHODS

Materials

Sucrose, sodium hydroxide (NaOH), trizma base (Tris), adenosine 5'-triphosphate disodium salt (Na_2ATP), nigericin, trichloroacetic acid (TCA), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), and (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethane-sulfonic acid]) (HEPES) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Carboxymethylcellulose (CMC) was obtained from Showa Chemical Co. (Tokyo, Japan). Formalin (17%) was obtained from Merck Co. (Darmstadt, Germany). Polyethyleneglycol 400 (PEG 400) and urethane were obtained from Junsei Chemical Co. (Tokyo, Japan). Sprague Dawley (SD) rats (male, 6 weeks, 180-250 g) were obtained from Charles River (Atsugi, Japan) and housed in constant conditions (23±2°C, 12 hr light) for 3~4 days before experiments.

Preparation of crude microsomes

Gastric microsomal fraction containing the H^+/K^+ ATPase was prepared as described previously (Cheon *et al.*, 1996). Stomach tissues from pig and New Zealand White rabbit (2.5~3.0 kg) were isolated, cut along the great curvature, washed with sterilized water

and kept in cold saturated NaCl for 30 min. The stomachs were washed three times with HEPES/sucrose buffer (2 mM HEPES, 250 mM sucrose, 2 mM $MgCl_2$, pH 7.4). Mucus layer was collected with slide glass and broken with 10 strokes in a glass homogenizer. The homogenate was centrifuged at 10,000×g at 4°C for 30 min, and supernatant was recentrifuged at 100,000×g for 1 hr. The resulting pellet was suspended in 40 mM Tris/HCl (pH 7.4) using 10 strokes in a glass homogenizer and aliquots of the preparation were stored at -70°C until use. This was used for the H^+/K^+ ATPase assay. The protein concentration was determined by the BioRad protein assay (Bradford, 1976).

H^+/K^+ ATPase assay

H^+/K^+ ATPase activity was determined as follows. Enzyme preparation (20 μg protein) was preincubated at 37°C water for 30 min in 200 μl of medium containing 40 mM Tris/HCl (pH 7.4) buffer, 4 mM $MgCl_2$, 5 μg/ml nigericin in methanol, and with or without synthetic compounds in DMSO. The reaction was initiated by adding 6.7 mM Na_2ATP (50 μl), continued for 30 min, and terminated by addition of 30% cold TCA (50 μl). The reaction mixture was centrifuged, and the released inorganic phosphate in the supernatant was measured spectrophotometrically (Yoda and Hokin, 1970). Specific H^+/K^+ ATPase activity was determined by the difference between the activities in the absence and in the presence of 48 mM KCl and 6 mM NH_4Cl.

Inhibition of basal acid secretion

Gastric anti-secretory activity was determined by Shay's method with some modification (Shay *et al.*, 1945). After fasting for 24 hr, pylorus of a SD rat was ligated under diethylether anesthesia, and then the compounds in 50% PEG 400 (5, 10, 20, 40 mg/kg) was administered intraduodenally. Five hour after surgery, the rat was killed by cervical dislocation. Following isolation of the stomach, gastric juice was collected, and after centrifugation at 5,000 rpm for 10 min, its volume was measured. An aliquot was used for determination of acid concentration by titration with 0.1N NaOH to an endpoint of pH 7.0 using the Orion 960 autochemistry analyzer (Boston, MA, U.S.A.). Acid output was calculated by multiplying the volume of gastric juice with the acid concentration.

Experimental ulcer models

Gastric mucosal lesions by ethanol and NaOH were produced according to the method of Robert *et al.* (1979). SD rats were fasted for 24 hr prior to the experiments with free access to water. AU-413 suspended in 0.5% CMC (30~300 mg/kg) was orally given 1 hr before oral administration of either 95% ethanol (1 ml/rat) or 0.3N NaOH (1 ml/rat). One hour later, the animals were killed by diethylether anesthesia, and the stomachs were isolated. After fixing the stomach in 13 ml of 1% formalin for 1 hr, the greater curvature of the stomach was opened. Macroscopical lesion was measured, summed up and compared with the group treated with either 95% ethanol alone or 0.3N NaOH alone. Control groups were treated with 0.5% CMC alone, followed by either 95% ethanol or 0.3N NaOH. All the group allocations of the experimental animals were done in a randomized order and under blind conditions.

Histamine-stimulated gastric acid secretion

For the lumen perfused rat studies, the method of Ghosh and Schild (Ghosh and Schild, 1958) was used. SD rat (10 weeks, male, 300~350 g) was fasted for 24 hr and anesthesized with *i.p.* injection of 1.2 g/kg urethane and tracheotomized. Polyethylene tube was inserted and fixed in the forestomach through esophagus, and another cannula was inserted into pyloric region of the stomach through duodenum. Animal was perfused with 37°C saline solution by using infusion pump at a flow rate of 1.5 ml/min. The perfusate from the duodenum tube was collected at 15 min interval, and determined for its volume and acid concentration by using Orion 960 autochemistry analyzer. Body temperature was maintained at 37°C by using an overhead heating lamp and an electric cushion. AU-413 suspended in 0.5% CMC was administered intraduodenally after the baseline was stabilized and histamine (2 mg/kg) in 0.9 % saline was *i.m.* injected as a secretagogue 30 min after the administration of the compound. The perfusate was collected for 2.5 hr after the administration of the compound. Percent inhibition was calculated from the comparisons of area under the curve of acid output in the presence or absence of AU-413 treatment.

Statistical analysis

Statistical evaluation of the results was performed using Student's *t*-test. A P value of <0.05 was regarded as statistically significant.

RESULTS

Effect of acylquinoline derivatives on H^+/K^+ ATPase activities

To evaluate anti-ulcer activities of newly synthesized acylquinoline derivatives, the effects of the synthetic compounds on gastric H^+/K^+ ATPase activities were examined. The structures of the acylquinoline derivatives

tested are shown in Fig. 1. As shown in Table I, some compounds such as AU-090, AU-091, AU-254, AU-413 and AU-466 had a moderate activity on both hog and rabbit enzyme inactivations. The effect of these compounds are comparable with the known anti-secretory compounds, omeprazole and SK&F96067. On the other hand, AU-085 and AU-293 showed potent inactivation on hog enzyme while they were relatively weak on rabbit enzyme. The reason for such differential effect is unknown at present.

Effect of acylquinoline derivatives on basal gastric acid secretion

To determine whether acylquinoline derivatives have *in vivo* anti-secretory activity, the effect on gastric acid secretion was determined using the Shay method (Shay *et al.*, 1945). As shown in Table II, AU-086, AU-291 and AU-413 had substantial *in vivo* anti-secretory activity although it is somewhat less potent

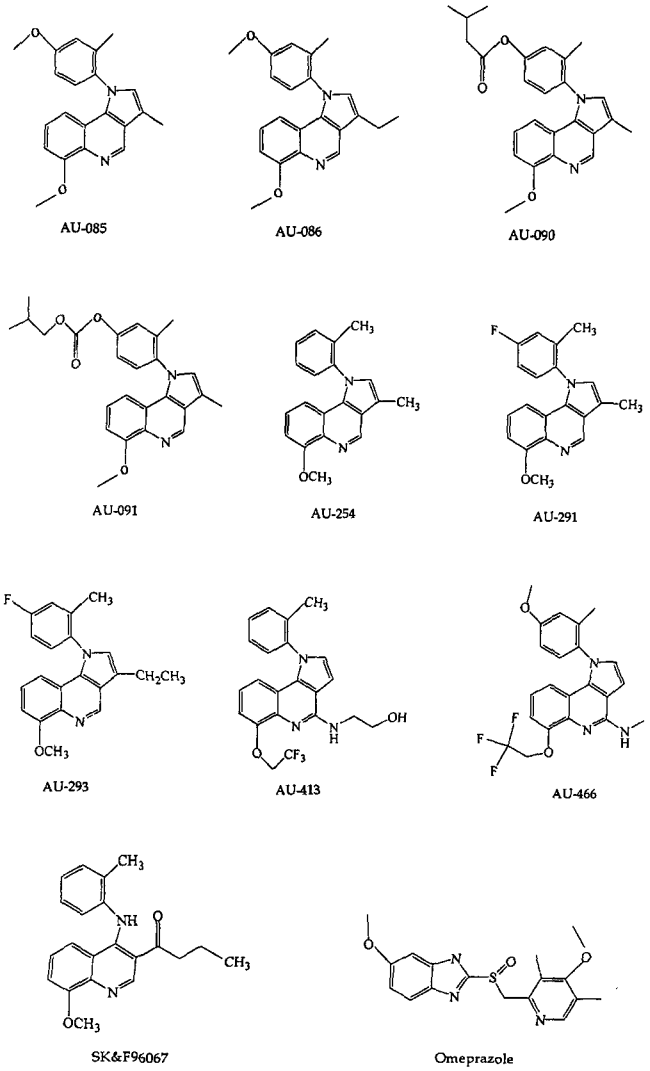

Fig. 1. Structures of acylquinoline derivatives.

Table I. IC_{50} values of acylquinoline derivatives

Compound	IC_{50} values (μM)[a]	
	Hog H^+/K^+ ATPase	Rabbit H^+/K^+ ATPase
AU-085	3.1	61.8
AU-086	25.7	52.1
AU-090	5.9	8.8
AU-091	16.0	23.7
AU-254	15.6	16.3
AU-291	53.8	30.0
AU-293	5.9	70.6
AU-413	8.1	34.6
AU-466	15.0	28.8
Omeprazole	9.6	22.4
SK&F96067	2.1	18.6

[a]Various concentrations of acylquinoline derivatives were incubated with either hog H^+/K^+ ATPase or rabbit H^+/K^+ ATPase. After 30 min incubation, the remaining activity was measured as described in Materials and Methods. Data represent the mean of two separate experiments.

Table II. ED_{50} values of acylquinoline derivatives on basal gastric acid secretion[a]

Compounds	ED_{50} value (mg/kg)
AU-085	40
AU-086	10
AU-090	>50
AU-091	>50
AU-254	35
AU-291	10
AU-293	13
AU-413	7.1
AU-466	45
Omeprazole	3.2
SK&F96067	15

[a]Acylquinoline derivatives (5, 10, 20, 40 mg/kg) were intraduodenally administered to rats under pylorus ligation, and stomach content was analyzed 5 hrs later. Data represent the mean of two separate experiments.

than omeprazole. It seems that the *in vivo* activity dose not exactly correlate with the *in vitro* H^+/K^+ ATPase inactivation. The inhibition of gastric acid secretion by these compounds appears to be associated with the decrease in the concentration as well as the volume of gastric juice.

Effect of acylquinoline derivatives on animal ulcer model

Based on the results obtained from *in vitro* and *in vivo* screening, AU-413 was the best candidate for further evaluation. Thus, the effect of AU-413 on experimentally induced ulcer was examined. In this study, we employed 95% ethanol and 0.3N NaOH as ulcer inducing agents. Oral administration of either 95% ethanol or 0.3N NaOH produced band like hemorrhagic lesion in grandular stomach. Ulcer lesion induced by either 95% ethanol or 0.3N NaOH was

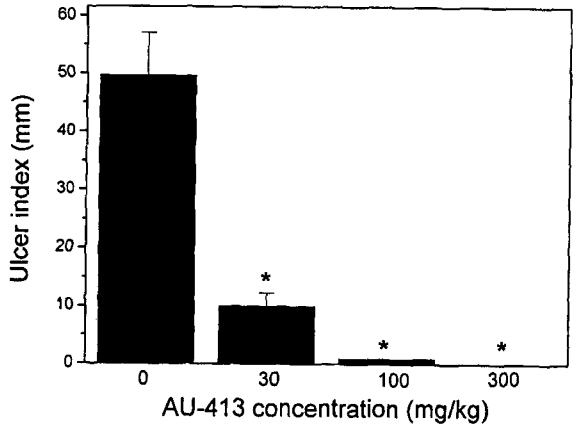

Fig. 2. Effect of AU-413 on ethanol-induced ulcer. AU-413 was given 1 hr before the administration of 1 ml of 95% ethanol. After isolation and fixation of stomach tissue, ulcer index was measured and summed up. Data represent the mean±SD (N=5). *P<0.05 vs control.

49.8±7.3 and 62.8±13.2 mm, respectively. As shown in Fig. 2, AU-413 produced dose-dependent protection from ethanol-induced gastric damage, significant protection being achieved at 30 mg/kg. Similarly, oral administration of AU-413 reduced gastric lesion caused by 0.3N NaOH (Fig. 3). ED_{50} values against each irritant

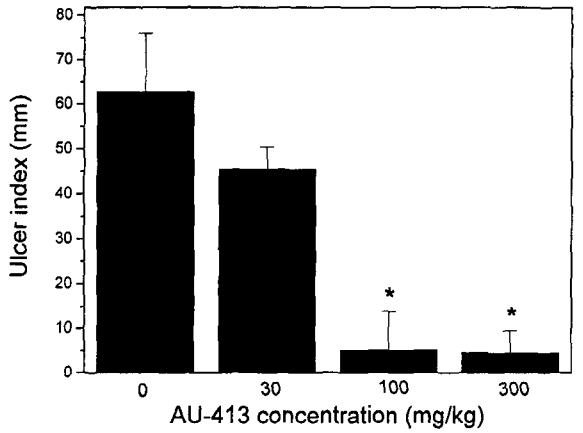

Fig. 3. Effect of AU-413 on NaOH-induced ulcer. AU-413 was given 1 hr before the administration of 1 ml of 0.3N NaOH. After isolation and fixation of stomach tissue, ulcer index was measured and summed up. Data represent the mean±SD (N=5). *P<0.05 vs control.

Table III. ED_{50} values of AU-413 in chemically-induced gastric ulcer[a]

Ulcer inducing agent	ED_{50} (mg/kg)
95% ethanol	12
0.3N NaOH	41

[a]Various concentrations of AU-413 were orally administered to SD rats and either 95% ethanol or 0.3N NaOH was orally given 1 hr later. The ulcer lesions were measured, summed up. Data represent the mean of two separate experiments.

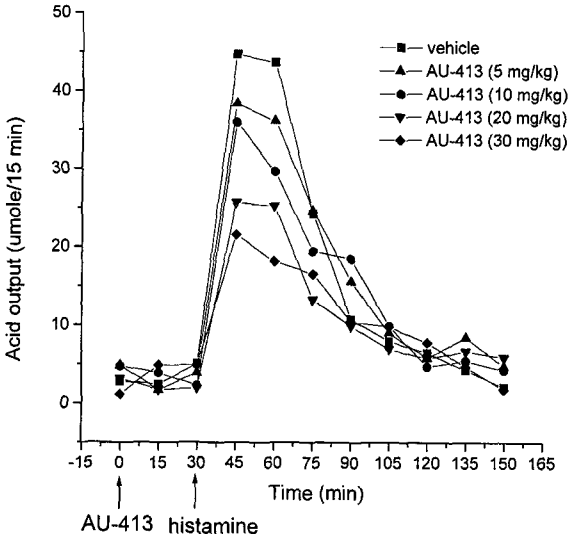

Fig. 4. Effect of AU-413 on histamine-stimulated gastric acid secretion. AU-413 (5, 10, 20, 30 mg/kg) was administered intraduodenally in the lumen perfused rat stomach preparation. Other experimental details were as described in Materials and Methods.

was presented in Table III.

Effect of AU-413 on histamine-stimulated gastric acid secretion

Besides the effect on basal acid secretion, AU-413 was further tested whether it shows anti-secretory activity on histamine-stimulated gastric acid secretion. As shown in Fig. 4, intraduodenal administration of AU-413 resulted in the reduction of histamine-stimulated gastric acid secretion. ED_{50} value was approximately 28 mg/kg.

DISCUSSION

Proton pump inhibitors have been considered to be important candidates for the development of new anti-ulcer agents since the discovery of omeprazole (Gustavsson *et al.*, 1983). The mechanism of action of omeprazole appears to be the activation to a reactive intermediate, which binds covalently to essential sulfhydryl group (s) of the H^+/K^+ ATPase (Lorentzon *et al.*, 1985; Keeling *et al.*, 1985). Thus, omeprazole inhibits the proton pump activity in an irreversible manner, resulting in the feedback increase of gastrin secretion. Indeed, issues on its safety have been raised such as enterochromaffin-like (ECL) cell carcinoid, hypergastrinemia and bacterial overgrowth due to long lasting anacidity (Larsson *et al.*, 1986; Konturek *et al.*, 1984).

Acylquinoline derivatives, for example, SK&F96067, have been reported to be reversible H^+/K^+ ATPase inhibitors (Pope and Parsons, 1993). We thought that

the reversible inhibitor of gastric H^+/K^+ ATPase would be beneficial in clinical use by overcoming the possible side effects of omeprazole. Thus, we designed several acylquinoline derivatives to develop novel anti-ulcer agents with less side effects.

In an initial *in vitro* screening, AU-090, AU-091, AU-254, AU-413 and AU-466 exhibited substantial inhibitory effects on both hog and rabbit H^+/K^+ ATPases. It appears that either 4-methoxy or 4-fluorine group attached to the pyrrolo ring does not improve the inactivation potency of above compounds on H^+/K^+ ATPase. However, in case of AU-085 and AU-293, they exhibited potent inactivation on the hog enzyme only. The exact explanation for this is not available, although it is possible that minor structural differences in the active site pockets of two enzymes contribute to the differential effects. With regard to the *in vivo* effect, AU-086, AU-291 and AU-413 appear to possess good anti-secretory activity when administered intraduodenally. Based on the observation that AU-090 and AU-091 had little in vivo activity despite good *in vitro* activity, the bulky group at 4 position of the phenyl ring attached to the pyrrolo structure does hinder the *in vivo* action. Under our experimental conditions, there was no good correlation between *in vitro* and *in vivo* activities. Possible explanations for this include species difference, and/or stability and metabolism of the compounds. In addition to the basal acid secretion, AU-413 inhibited histamine-stimulated gastric acid secretion. It is known that histamine stimulates gastric acid secretion by binding to the histamine H_2 receptor, and subsequently activates the proton pump, thereby secreting gastric acid (Sachs, 1986). Under our experimental conditions, the action of *i.m.* administration of histamine (2 mg/kg) lasts about 1.5 hr, and AU-413 reduced total acid output induced by histamine with an ED_{50} value of 28 mg/kg.

Considering the *in vitro* and *in vivo* results together, we selected AU-413 as a candidate for further investigation. AU-413 protected gastric damage induced by either ethanol or NaOH in a dose dependent manner. The protective effect against ethanol injury was ascribed to the cytoprotective property of the compound (Guth, 1982). The cytoprotection is due to the secretion of bicarbonate and/or mucus, production of nitric oxide and/or prostaglandins, stimulation of the sodium pump and so on. Detailed studies on the cytoprotective mechanism of AU-413 are currently in progress. Results on the compounds having both anti-secretory and cytoprotective activities have been reported previously (Long *et al.*, 1983; Kurebayashi *et al.*, 1988; Oh *et al.*, 1997).

In summary, a representative acylquinoline derivative AU-413 inhibits gastric H^+/K^+ ATPase isolated from either pig or rabbit. In addition, gastric acid secretion was blocked upon AU-413 administration. Using chemically-induced animal ulcer model, AU-413 protected gastric damage in a dose dependent manner. Based on the present study, AU-413 is a suitable candidate for further characterization including structural optimization, pharmacokinetics and toxicity studies.

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