Syntheses and Inhibitory Effects on Gastric Lesions of *trans*-Guanidinomethylcyclohexane Carboxylic Acid Arylamides

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A novel series of *trans*-guanidinomethylcyclohexanecarboxylic acid (*trans*-GMCHA) arylamides was synthesized. The several *trans*-GMCHA arylamide derivatives showed more potent inhibitory effects on the stress- and HCl–ethanol-induced gastric ulcers than cetraxate in rats. In acute toxicity studies in mice, most amides showed such severe toxicity that all mice injected with these compounds (50 mg/kg, i.p.) died. However, mice injected with the *trans*-GMCHA (2′-,3′- and 4′-ethoxycarboxy)phenylamide (7, 8 and 9) which bear an alkyloxycarbonyl group at benzene ring survived.

From these results, *trans*-GMCHA (2′-ethoxycarbonyl)phenylamide (7) was selected as a promising anti-ulcer agent.

Keywords *trans*-GMCHA; *trans*-GMCHA (2′-ethoxycarboxy)phenylamide; anti-ulcerous activity; anti-serine proteases activity

We previously reported work on the synthesis of a series of *trans*-4-guanidinomethylcyclohexanecarboxylic acid (*trans*-GMCHA) arylesters, in which these compounds were tested for inhibitory effects on several kinds of serine-proteases and for pharmacological effects.[1] The *trans*-GMCHA-4′-*tert*-butylphenyl ester (**1**) exhibited strong anti-allergic effect and the *trans*-GMCHA 2′-benzyloxy-carbonylphenyl ester (**2**) demonstrated anti-ulcerous activity, although both strongly inhibited chymotrypsin.

As the mechanism of the anti-allergic effect of **1**, Muramatu *et al.*[2] stated that **1** strongly and characteristically inhibited tryptase (trypsin-like enzyme) which existed in the mast cells and was one of the trigger enzymes causing histamine-release from the cells. The β-cyclodextrin clathrate of **2** (benexate hydrochloride, betaclex) has wide anti-ulcerous spectra and is used for the treatment of ulcers in Japan. The modes of anti-ulcerous action of benexate are thought to improve the blood flow of the microcirculation system on stomach mucosae and to keep the level of endogenous prostaglandins.[3] These modes of action are similar to that of cetraxate, a typical potentiation of defensive factors of anti-ulcerous agent.[4,5]

Although benexate and cetraxate, which are a series of ω-aminoalkylcarboxylic acid arylesters, have an inhibitory effect on serine proteases, these inhibitory effects were proposed not to influence their anti-ulcerous activity[1] even though they caused the exacerbation of intestinal hemorrhage in digestive tracts.[6] Therefore, we designed and synthesized a novel series of *trans*-GMCHA arylamides in order to obtain compounds having anti-ulcerous activity and less inhibitory effect on serine proteases. In this report, we discuss the structure–activity relationships toward serine-proteases and the anti-ulcerous activities, and add a the toxicological assessment.

Materials and Methods

General All melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were measured with a Shimadzu IR-27G. The mass spectra (MS) were recorded on a Shimadzu GC-MS 9000B. All elemental analyses were found to be within 0.4% of the calculated values.

Materials The aniline compounds were purchased from commercial sources. (*trans*-GMCHA)hydrochloride was synthesized according to the method described.[1] Cimetidine was kindly supplied by Dr. Endo, Department of Pharmacology of our university. Trypsin (from bovine pancreas, lot. no. 37c-0423-1) and elastase type III (from porcine pancreas, lot. no. 94f-80501) were purchased from Sigma Chemical Co., St. Louis. Benzoyl-DL-arginine-*p*-nitroanilide (BAPA) was from the Peptide Institute Inc., Osaka.

Synthesis of *trans*-GMCHA Arylamides *trans*-GMCHA hydrochloride (1.00 g, 4.2 mmol), corresponding aniline (4.0—4.5 mmol) and *N*,*N*′-dicyclohexylcarbodiimide were dissolved in dry pyridine (20 ml), and the solution was stirred for 40 h at room temperature. After 10 ml of H_2O was added, the mixture was stirred for 30 min. The precipitate was removed by filtration, the filtrate was evaporated and the residue was dried *at vacuo*. The crystals obtained were recrystallized from H_2O, and thus the corresponding *trans*-GMCHA arylamide hydrochloride was obtained.

Assay Methods of Trypsin and Elastase Activities The effects of *trans*-GMCHA arylamides on trypsin activity were determined as follows.[7] Reaction mixtures contained 1 mM BAPA and trypsin in 0.1 M borate buffer, pH 8.0, contained 0.01 M $CaCl_2$ at 25°C. The absorption intensity of reaction mixture was measured at 410 nm by spectrophotometer (Hitachi 150-20).

The effects of the arylamides on elastase activity were determined as follows.[8] Elastase solution was added to the elastin–congo red (5 mg) suspension in 0.05 M Tris–HCl buffer, pH 8.8 (3.5 ml). The test tube was slowly shaken for 30 min at 37 °C. After centrifugation at 3000 rpm for 5 min, the absorption of the supernatant was measured at 495 nm by spectrophotometer.

Assay for Anti-ulcer Activity Gastric ulcers induced by stress in rats were caused using the method described by Takagi and Okabe.[9] Namely, after 24 h fasted condition, male Sprague-Dawley rats (200—250 g) were immersed in a water bath (23 ± 1 °C) as far as the xiphoid process, and killed by ether anesthesia after 16 h of stress load.

HCl–ethanol-induced gastric ulcers were caused by the method of Mizui and Doteuchi.[10] Male Sprague-Dawley rats (200—250 g) were fasted for 24 h before the experiments, but allowed free access to water. A concentration of ethanol (60%) in 150 mM HCl was given orally in 1 ml injection, and the animals were sacrificed with an overdose of ether 1 h later.

Test compounds were suspended in 0.5% sodium carboxymethyl cellulose solution and given orally 20 min before the stress load or 30 min before the HCl–ethanol administration.

The stomachs were removed and inflated by pouring into them 10 ml of 1% formalin which was left for 10 min to fix the gastric wall. Subsequently, the stomachs were incised along the greater curvature and examined for lesions. The length (mm) of each lesion was measured under a dissecting microscope (× 10) with a square grid, and the sum per stomach indicated the lesion index.

Intestinal Hemorrhage The method of intestinal hemorrhage was similar to an HCl–ethanol-induced gastric ulcer. The length (mm) of hemorrhage was measured, and summed per intestine to indicate the intestinal hemorrhage.

Acute Toxicity Testing The *trans*-GMCHA arylamides, dosage of 0 (vehicle), 50, 100, 300 and 600 mg/kg for i.p. and 1000, 4000 and 8000 mg/kg for *p.o.* were administered to each group a single time. Each dosage group consisted of both sexes of ICR strain mice 5 weeks old when the study began. The animals were observed for 14 d after dosing. Clinical observation and mortality check were made shortly after dosing, at frequent intervals over 6 h, and once each morning and afternoon thereafter.

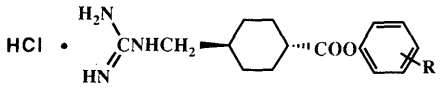
Results and Discussion

Syntheses of *trans*-GMCHA Arylamides The syntheses of a series of the *trans*-GMCHA arylesters containing **1** and **2** as new serine protease inhibitors were done in our laboratory.[1] The chemical structures of these compounds (**1**, **2**) are shown in Chart 1. A novel series of compounds (**3**—**9**, listed in Chart 2) was synthesized according to a previous paper.[1] *trans*-GMCHA hydrochloride and corresponding aniline were condensed in pyridine by *N*,*N*′-dicyclohexylcarbodiimide to yield *trans*-GMCHA arylamide hydrochloride. The structure of these compounds was determined by elemental analysis, infrared spectrum, and MS. These results are summarized in Table I along with the yield of each compound.

Inhibitory Effects of *trans*-GMCHA Arylamides on the Serine Proteases The effects of **4**, **5**, **7**, **8** and **9** were investigated on trypsin and elastase obtained from bovine pancreas. These results (IC_{50} (μM) values) determined by assays are shown in Table II. That is, the ester compound **2** showed the considerably potent inhibitory activity on both trypsin and elastase, whereas the amides showed inhibition 8—35 times and 2—4 times less potent than **2**, respectively. Okano *et al.*[11] reported that *trans*-aminomethylcyclohexane carboxylic acid arylamides caused a more drastic decrease of the anti-plasmine activity than ester types. These findings taken together suggest that the ester part of ω-aminoalkylcarboxylic acid is necessary or at least suitable for anti-serine protease activities. We thought that the novel amide compounds would not cause the exacerbation of intestinal hemorrhage because their anti-serine protease activity was depressed.

Effects of the *trans*-GMCHA Arylamides on the Gastric Lesions The effects of **3**—**9**, cetraxate, benexate and cimetidine were assessed on gastric lesions induced by water-immersion stress in rats (Table III). Compounds **3**—**7** exhibited an inhibitory effect of over 50% at 100 mg/kg *p.o.* on the gastric lesions, and their activities were significantly stronger than those of cetraxate and benexate. From these results, it is clear that the inhibitory activities of these amides on the serine proteases have no or little correlation to their anti-ulcerous action, though the anti-ulcer agents such as cetraxate and benexate had the ordinary design of serine protease inhibitors. It was suggested that the anti-serine protease activity was not requisite to these anti-ulcerous agents.

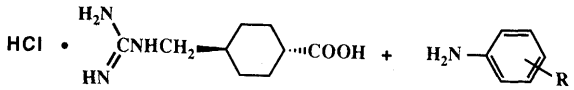
In acute toxicity studies of **3**—**9** in mice, **4**, **5** and **6** showed such severe toxicity that all mice injected with these compounds (50 mg/kg, i.p.) died; mice injected with **7**, **8** and **9** which bear an alkyloxycarbonyl group at the benzene ring, however, survived. Compound **7**, which exhibited the strongest anti-ulcerous activity among **7**—**9**, was therefore selected for further pharmacological and toxicological

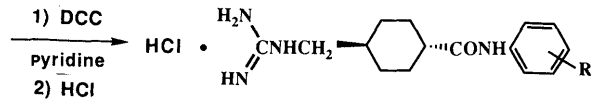


1: R=4-*tert*-Bu

2: R=2-$COOCH_2Ph$

Chart 1





3: R=4-NO_2 **7**: R=2-$COOC_2H_5$

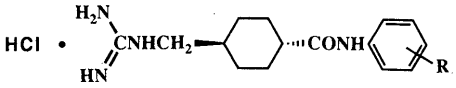
4: R=4-CH_3 **8**: R=3-$COOC_2H_5$

5: R=4-Cl **9**: R=4-$COOC_2H_5$

6: R=4-*tert*-Bu

Chart 2

TABLE I. Physical Constants and Spectral Data for *trans*-GMCHA Arylamides



No.	R	Yield (%)	mp (°C)	IR ν_{max}^{KBr} cm^{-1}			MS *m/z* (M^+ − HCl)	Formula	Analyses[a]
				H_2N HN =CNH–	–CONH–	–COOR–			
3	4-NO_2	49	>275	3401	1655		319	$C_{15}H_{21}N_5O_3 \cdot HCl$	C, H, N
4	4-CH_3	32	142—146	3246	1645		288	$C_{16}H_{24}N_4O \cdot HCl$	C, H, N
5	4-Cl	63	169—170	3389	1645		308, 310[b]	$C_{15}H_{21}ClN_4O \cdot HCl$	C, H, N
6	4-*tert*-Bu	12	172—178	3333	1650		330	$C_{19}H_{30}N_4O \cdot HCl$	C, H, N
7	2-COOEt	66	91—92	3367	1655	1670	346	$C_{18}H_{26}N_4O_3 \cdot HCl$	C, H, N
8	3-COOEt	55	264—266	3367	1661	1712	346	$C_{18}H_{26}N_4O_3 \cdot HCl$	C, H, N
9	4-COOEt	80	133—135	3333	1678	1698	346	$C_{18}H_{26}N_4O_3 \cdot HCl$	C, H, N

a) All elemental analyses were found to be within 0.4% of calculated values. *b*) $M^+ + 2$.

TABLE II. Anti-serine Protease Activities of *trans*-GMCHA Arylamides and *trans*-GMCHA-2′-benzyloxycarbonylphenyl Ester **2**

Compound	Trypsin IC_{50} (μM)	Elastase IC_{50} (μM)
2	82	60
4	2797	234
5	1123	126
7	736	—[a]
8	661	200
9	1131	136

a) Could not be determined because of coloring of the test solution in the absence of the substrate.

TABLE III. Effects of *trans*-GMCHA Arylamides on Stress-Induced Gastric Lesions in Rats

Compound	Dose (mg/kg *p.o.*)	Ulcer index (mm)[a]	Inhibition (%)
Vehicle		16.7±1.7	
2	200	13.4±3.3	20
3	100	7.9±2.8	53
4	100	4.3±1.7	74
5	100	1.4±0.6	92
6	100	4.0±1.5	76
7	100	3.5±2.2	79
8	100	20.1±4.4	−20
9	100	15.1±3.2	10
Cimetidine	100	2.2±0.5	85
Cetraxate	100	10.7±3.0	36

a) Each value represents the mean±S.E., $n=5$—10.

studies.

The acute toxicity of **7** at various doses is shown in Table IV; the minimum lethal dose was over 4000 mg/kg *p.o.*

As can be seen in Table III, the inhibitory activity of **7** toward the stress-induced ulcer was almost equal to that of cimetidine; however, this compound did not show any effect on gastric acid secretion (data not shown). Examination of the inhibitory effect of **7** on HCl–ethanol-induced ulcer revealed that the compound showed more potent anti-ulcerous activity in rats than cetraxate (Table V). Compound **7** did not demonstrate any intestinal hemorrhage, however, which was the typical adverse effect observed in a serine protease inhibitor like cetraxate (Table V). From these data, **7** is presumed to be a cytoprotective type anti-ulcerous agent. Our conclusion is that **7** is a novel lead compound for therapy of a hemorrhaging ulcer.

TABLE IV. Acute Toxicity of **7** in ICR Male Mice

Dose (mg/kg)	Route	Survivors/total[a]
50	i.p.	2/2
100	i.p.	2/2
300	i.p.	2/2
600	i.p.	2/2
1000	*p.o.*	2/2
4000	*p.o.*	5/5
8000	*p.o.*	4/5

a) Mice surviving at 2 d and total mice used in test.

TABLE V. Effects of **7** and Cetraxate on HCl–EtOH Induced Gastric Lesions and Intestinal Hemorrhage in Rats

Treatment	Dose (mg/kg *p.o.*)	Ulcer index[a] (mm)	Inhibition (%)	Intestinal hemorrhage (mm)
Control		110.9±31.8		2.8
Cetraxate	100	49.6±10.1	55.3	4.5
7	100	23.5± 5.6	78.8	1.4

a) Mean±S.D., $n=6$.

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