Effect of Methylcarbonylmethyl 2(S)-[4-(4-Guanidino-benzyloxy)phenyl] Propionate Methanesulphonate (TT-S24) on Experimental Pancreatitis in Rats

Hiroyuki Tagami,*a,b Shin-ichi Abe,*a Yasuko Yoshida,*a Ikuo Tanaka,*a and Chiaki Kamei*b

Teikoku Chemical Industries Co., Ltd.,*a 5-41 Senzo, Itami, Hyogo 664, Japan and Department of Pharmacology, Faculty of Pharmaceutical Sciences, Okayama University,*b 1-1-1 Tsushima-naka, Okayama 700, Japan.
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The effect of methylcarbonylmethyl 2(S)-[4-(4-guanidino-benzyloxy)phenyl] propionate methanesulphonate (TT-S24) on experimental pancreatitis in rats was examined in comparison with that of camostat. TT-S24 showed a preventive effect on increases in plasma amylase activity and pancreatic weight induced by cerulein injection. TT-S24 also reduced an increase in plasma amylase activity induced by taurocholate. TT-S24 effectively prevented the mortality induced by an injection of a mixture of trypsin and taurocholate. TT-S24 showed no effect on an increase in amylase activity 6 h after duodenum ligation (closed duodenal loop pancreatitis), indicating that the drug had no effect on the initiation and propagation step of closed duodenal loop pancreatitis. On the other hand, TT-S24 reduced an increase in amylase activity 6 h after release of the duodenum ligation. TT-S24 showed anti-trypsin, anti-kallikrein, anti-thrombin and anti-plasmin activities. The effect of TT-S24 on some experimental pancreatitis models was nearly equal to or somewhat more potent in most instances to that of camostat. Therefore, TT-S24 should be useful in the clinical treatment of pancreatitis.

Key words guanidinobenzoate derivative (TT-S24); experimental pancreatitis; trypsin inhibitor; thrombin inhibitor; camostat

Pancreatitis can be classified into two major types, i.e., acute pancreatitis and chronic pancreatitis. The major symptoms of acute pancreatitis include an increase in plasma amylase and lipase activities, hypovolaemia, hypoalbuminemia, pulmonary oedema and abdominal pain; in a mild case, acute pancreatitis is cured completely; however, in a severe case, the disease can cause multiple organ failure. On the other hand, chronic pancreatitis progresses, repeatedly showing symptoms of acute pancreatitis until it reaches the point of irreversible destruction of the pancreas.

It is generally recognized that intrapancreatic digestive enzyme activation is an important early event in the pathogenesis of acute pancreatitis. Among those activated digestive enzymes, trypsin, which can activate other digestive enzymes as well as itself, is assumed to be a key enzyme in pancreatitis. At the present, several drugs have been used for the treatment of acute pancreatitis, most of which, for example, gabexate mesilate and aprotinin, are trypsin inhibitors. However, for the treatment of chronic pancreatitis, only camostat, which is also a trypsin inhibitor, has been used clinically. Many attempts have been made to develop new drugs for the treatment of acute pancreatitis and/or chronic pancreatitis.

Methylcarbonylmethyl 2(S)-[4-(4-guanidino-benzyloxy)phenyl] propionate methanesulphonate (TT-S24) is a new trypsin inhibitor synthesized by Teikoku Chemical Industries Co., Ltd. (Fig. 1). In this paper, the effect of TT-S24 on several types of experimental pancreatitis models was studied in comparison with that of camostat.

MATERIALS AND METHODS

Animals Male Wistar rats (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed for a week before the experiments (light dark cycle 12 h, room temperature 22—25 °C, relative humidity 55±5%). The body weights of the rats were 180—220 g at the beginning of the experiments.

Materials TT-S24 was synthesized at Teikoku Chemical Industries Co., Ltd., and the following chemicals were used in the study: camostat (Sumika Fine Chem), cerulein (Kyowa Hakko), sodium taurocholate (Difco), amylase test kit Wako (Wako), trypsin (Sigma), thrombin (Sigma), plasminogen (Sigma), kallikrein (Sigma), streptokinase (Lederle), benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPA, Peptide Institute), BocValProArgMCA (Peptide Institute), BocValLeuLysMCA (Peptide Institute), ProPheArgMCA (Peptide Institute) and SucAlaProAlaMCA (Peptide Institute).

Effect on Cerulein-Induced Pancreatitis The experiment was performed according to the method of Otsuki, with minor modification. The animals used were 8—30 rats for each group. Cerulein pancreatitis was induced by subcutaneous injections of 20 µg/kg of cerulein at hourly intervals, 4 times over 3 h. At 6 h after the first cerulein injection, blood was collected from the jugular vein under light ether anaesthesia.
sia for measuring plasma amylase activity. After blood collection, rats were sacrificed by an excess of ether inhalation, and the pancreas was removed and weighed. Drugs were administered orally 3 h before the first injection of cerulein. TT-S24 and camostat were suspended in 0.5% methylcellulose. For a control group, 0.5% methylcellulose was administered.

**Effect on Taurocholate-Induced Pancreatitis** The experiment was performed according to the modified method of Lankisch et al. The animals used were 8–30 rats for each group. Taurocholate-induced pancreatitis was induced by retrograde injection of 0.1 ml of 5% sodium taurocholate into the pancreas through the pancreatic duct with a polyethylene tube (PE10) under light ether anesthesia. When sodium taurocholate was injected, the common bile duct was clamped at the liver hilus. At 3 and 6 h after the injection of sodium taurocholate, the blood was collected from the jugular vein under light ether anesthesia for measuring plasma amylase activity. Drugs were administered orally twice, at 12 h and at 3 h before the injection.

**Effect on Pancreatitis Induced by a Mixture of Trypsin and Taurocholate** The experiment was performed according to the method of Murakami et al., with minor modification. Twenty rats which had been fasted for 12 h were used for one group. After obstruction of the common bile duct at the liver hilus with a clamp, pancreatitis was induced by retrograde injection of 0.1 ml of a mixture of 0.03 mg of trypsin and 10 mg of sodium taurocholate under light ether anesthesia. Immediately after the injection of this mixture, the pancreatic duct was ligated at the end of the duct near the duodenum. Survival was counted between 15 and 24 h after the injection, and the survival ratio was calculated. Drugs were administered orally 1 h before the induction of pancreatitis.

**Effect on Pancreatitis Induced by a Closed Duodenal Loop (CDL Pancreatitis)** The experiment was performed according to the method of Nevalainen et al. Nine rats which had been fasted for 12 h were used for each group. Under light ether anesthesia, a closed duodenal loop was created by ligating the duodenum at 2 points, on both the oral and anal sides of the orifice of the common bile duct. Blood was collected 3 times for measurement of plasma amylase activity: just before the duodenum ligation, 6 h after the duodenum ligation, and 6 h after releasing the duodenum ligation. TT-S24 at a dose of 100 mg/kg was administered orally 1 h before the duodenum ligation.

**Measurement of Plasma Amylase Activity** A blood sample was mixed with a 10% volume of 3.8% citrate, then centrifuged at 3000 rpm for 5 min. After centrifugation, a plasma sample was obtained. The plasma sample was diluted with saline to an adequate concentration, and the amylase activity was measured with Amylase test kit Wako. Plasma amylase activity was expressed in Caraway units (C.U.).

**Measurement of Inhibitory Potency to Enzymes** The inhibitory effect of drugs on trypsin activity was performed according to the method of Erlanger, with an absorption method, whereas the effects of drugs on thrombin, plasmin, kallikrein and elastase activities were performed according to the method of Tamura et al., using the fluorescence method. Experimental conditions were shown below. Trypsin: Substrate 1 nmol BAPA, reaction temperature 25°C, reaction time 10 min. Thrombin: Substrate 10 μM BocValProArgMCA, reaction temperature 37°C, reaction time 10 min. Plasmin: Substrate 30 μM BocValLeuLysMCA, reaction temperature 37°C, reaction time 30 min. Kallikrein: Substrate 20 μM ProPheArgMCA, reaction temperature 37°C, reaction time 20 min. Elastase: Substrate 40 μM SucAlaProAlaMCA, reaction temperature 37°C, reaction time 10 min. Plasmin was obtained from plasminogen activated by streptokinase. All reactions were stopped by the addition of 30% AcOH. After stopping the reaction, the absorbance at 410 nm for trypsin, and the fluorescence intensity at 440 nm with 380 nm excitation wavelength for thrombin, plasmin, kallikrein and elastase were measured. After measurement of the absorbance or fluorescence intensity, the IC50 value was calculated.

**Statistical Analysis** Data were expressed as the mean ± S.E.M. Statistical analysis was performed by ANOVA with Dunnett's test. In the experiment involving pancreatitis induced by a mixture of trypsin and taurocholate, the χ square test was used.

**RESULTS**

**Effect on Cerulein-Induced Pancreatitis** The effects of TT-S24 on cerulein-induced pancreatitis are shown in Fig. 2. Plasma amylase activity (control group) was increased by the subcutaneous injection of cerulein 5.7 times compared with normal group (normal group 1166±29 C.U., n=30, control group 6665±548 C.U., n=20). TT-S24 showed a marked attenuation of the cerulein-induced increase in plasma amylase activity. Significant effects were observed with 3 and 30 mg/kg p.o. Camostat at a dose of 30 mg/kg p.o. also showed a significant inhibitory effect; however, the potency was less than that of TT-S24. Pancreatic weight was also increased by cerulein injection (control group), 1.6 times compared with the normal group (normal group 0.75±0.03 g, n=8, control group 1.22±0.08 g, n=20). TT-S24, as well as camostat, showed an inhibitory effect on the increase in pancreatic weight. A significant effect was observed with 30 mg/kg p.o.

![Fig. 2. Effect of TT-S24 on Increases in Plasma Amylase Activity and Pancreatic Weight Induced by Cerulein](image-url)

(A) plasma amylase activity, (B) pancreatic weight. Each data represents mean ± S.E.M. (n=8–30). †: significantly different from control group with p<0.01.
of both drugs, but the effect of TT-S24 was slightly more potent than that of camostat.

Effect on Taurocholate-Induced Pancreatitis The results are shown in Fig. 3. Plasma amylase activity was increased by taurocholate (control group) 6.2 times compared with the normal group at 3 h after taurocholate injection (normal group 1166±29 C.U. n=30, control group 7227±356 C.U. n=30), and 8.1 times compared with the normal group at 6 h after taurocholate injection (normal group 1166±29 C.U. n=30, control group 9478±378 C.U. n=30). TT-S24 at doses of 30 and 100 mg/kg p.o. caused a significant inhibition of the increase in plasma amylase activity at both 3 and 6 h after taurocholate injection. Almost the same effect was observed with camostat.

Effect on Pancreatitis Induced by a Mixture of Trypsin and Taurocholate The survival rate of rats with experimental pancreatitis is shown in Fig. 4. In the control group, 60% of the rats survived 15 h after the induction of pancreatitis, but by 21 h after, all the animals had died. The oral administration of TT-S24 caused a dose-dependent decrease in mortality, and a significant effect was observed with 100 mg/kg p.o. 16, 17, 18 and 19 h after the induction of pancreatitis compared with the control group. Camostat also showed a similar effect, but no significant effect was observed, even at a dose of 100 mg/kg p.o.

Effect on CDL Pancreatitis The results are shown in Fig. 5. Plasma amylase activity in the control group was increased 6 h after the duodenal ligation and 6 h after releasing the duodenal ligation compared with that observed before ligation. At 6 h after ligation, TT-S24, as well as camostat, showed no significant inhibitory effect on the increase in amylase activity induced by CDL. At 6 h after CDL release, the oral administration of TT-S24 at a dose of 100 mg/kg p.o. caused a significant preventive effect on the increase in plasma amylase activity. Camostat at a dose of 100 mg/kg p.o. also showed a similar effect.

Inhibitory Potency to Enzymes The results are shown in Table 1. TT-S24 caused anti-trypsin and anti-kallikrein activity at the same potency as camostat. TT-S24 also showed anti-thrombin and anti-plasmin activities. These effects of TT-S24 were more potent than those of camostat. However, no significant difference was observed between TT-S24 and

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**Fig. 3.** Effect of TT-S24 on an Increase in Plasma Amylase Activity Induced by Taurocholate

(A) 3 h after taurocholate injection, (B) 6 h after taurocholate injection. Each data represents mean±S.E.M. (n=8—30). *: significantly different from control group with p<0.05, **: significantly different from control group with p<0.01, respectively.

**Fig. 4.** Effect of TT-S24 on Mortality Induced by Trypsin and Taurocholate Injection

○, control; ●, TT-S24; △, camostat. (A) 30 mg/kg, p.o., (B) 100 mg/kg p.o. *: significantly different from control group with p<0.05.

**Fig. 5.** Effect of TT-S24 on an Increase in Amylase Activity Induced by CDL

▱, before ligation; ■, 6 h after ligation; △, 6 h after CDL release. *: significantly different from the value 6 h after ligation with p<0.05 and p<0.01, respectively.

**Table 1.** IC$_{50}$ Values for TT-S24 and Camostat on the Inhibition of Some Enzyme Activities

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>TT-S24 (×10$^{-7}$ M)</th>
<th>Camostat (×10$^{-7}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>2.3 (1.1—4.8)</td>
<td>2.7 (1.2—6.2)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>3.2 (1.0—9.9)</td>
<td>7.4 (2.8—19.2)</td>
</tr>
<tr>
<td>Plasmin</td>
<td>0.18 (0.07—0.47)</td>
<td>0.66 (0.24—1.78)</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>110 (20—510)</td>
<td>110 (30—450)</td>
</tr>
<tr>
<td>Elastase</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
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</table>
camostat in inhibiting thrombin or plasmin activities. On the other hand, TT-S24 and camostat showed no inhibitory effect on elastase activity.

DISCUSSION

In the present study, it was found that TT-S24 prevented an increase in plasma amylase activity and pancreatic weight induced by cerulein. The potency of TT-S24 was greater than that of camostat, especially in plasma amylase activity. Otsuki et al. also reported that the oral administration of camostat (100 mg/kg) significantly prevented the cerulein-induced increase in plasma amylase activity. In addition, they demonstrated that the increased pancreatic weights were significantly reduced by camostat. In our present study, no cases of death were observed, and bleeding or necrosis was also rarely seen in the pancreas by microscopic observation after the subcutaneous injection of cerulein. From these findings, cerulein-induced pancreatitis reported in the present study was regarded as an experimental model for light acute pancreatitis. It seems likely, therefore, that TT-S24, as well as camostat, is expected to clinically inhibit light acute pancreatitis.

It is thought that pancreatitis is induced by an adverse current of bile from the duodenum to the pancreas through the pancreatic duct. Taurocholate-induced pancreatitis is an experimental model developed according to this hypothesis. Some investigators have reported that taurocholate-induced pancreatitis is an experimental model for severe acute pancreatitis. In our experimental condition, although no cases of death were observed, bleeding in macro- and microscopic observation and necrosis in microscopic observation were seen. Therefore, it is reasonable to presume that taurocholate-induced pancreatitis under our experimental condition might be an acute pancreatitis model of middle grade severity. TT-S24 showed an inhibitory effect on taurocholate-induced pancreatitis, and the potency was almost the same as that of camostat. Therefore, TT-S24 was expected to show an effect on middle grade severity of acute pancreatitis when used clinically.

As shown in Fig. 4, all rats were dead at 21 h after the injection of a mixture of trypsin and taurocholate. The pancreatitis induced by injection of this mixture of trypsin and taurocholate was regarded as an experimental model of severe acute pancreatitis. With the progressive destruction of the pancreas is severe acute pancreatitis, pancreatic enzymes, for instance, trypsin, phospholipase A2 and lipase, leaked into the blood flow. These enzymes activate the coagulation system, fibrinolytic system, kallikrein kinin system or other systems, and induced multiple organ failure which occasionally results in death. TT-S24 effectively prevented the mortality induced by an injection of this mixture of trypsin and taurocholate. This effect of TT-S24 was more potent than that of camostat. It was found that TT-S24 had more potent inhibitory effects on thrombin activity than camostat. As shown in the text, TT-S24 showed an inhibitory effect on the pancreatitis induced by injection of the trypsin and taurocholate mixture, and this effect was more potent than that of camostat. In the pancreatitis due to a mixture of trypsin and taurocholate, the leakage of digestive enzymes into the blood flow resulted in an activation of thrombin activity; consequently, it influences the condition of the whole body to be worse. Therefore, it seems likely that the potent effect of TT-S24 on pancreatitis induced by a mixture of trypsin and taurocholate is related to its stronger inhibition of thrombin activity.

The reflux of bile and pancreatic enzymes secreted into the duodenum are important early events in the initiation of acute pancreatitis. A similar mechanism is presumed to act in the occurrence of CDL pancreatitis. TT-S24, as well as camostat, caused no inhibition of the initiation and propagation steps in CDL pancreatitis; however, these drugs facilitated the recovery step. It is well known that chronic pancreatitis develops by the repeated occurrence of symptoms of acute pancreatitis. TT-S24 is expected to have a restorative effect on chronic pancreatitis, similar to camostat, because TT-S24 facilitated the recovery step of chronic pancreatitis. In conclusion, the effect of TT-S24 on some experimental pancreatitis models was nearly equal to or somewhat more potent than that of camostat in most instances. It seems, therefore, worthwhile to study the clinical efficacy of this compound in the treatment of pancreatitis in man.

REFERENCES