Inhibitory Effects of ONO-3307 on Various Proteases and Tissue Thromboplastin In Vitro and on Experimental Thrombosis In Vivo

Syozo MATSUOKA, Mayumi FUTAGAMI, Hiroyuki OHNO, Katsuhiko IMAKI, Tadeo OKEGAWA and Akiyoshi KAWASAKI
Minase Research Institute, Ono Pharmaceutical Co., Ltd., Osaka 618, Japan

Accepted August 10, 1989

Abstract—The effect of ONO-3307 (4-sulfamoyl phenyl-4-guanidinobenzoate methanesulfonate), a new protease inhibitor, was studied on various proteases in vitro and in an experimental thrombosis model in vivo. ONO-3307 competitively inhibited trypsin, thrombin, plasma kallikrein, plasmin, pancreatic kallikrein and chymotrypsin; and their \( K_i \) values were 0.048 \( \mu \)M, 0.18 \( \mu \)M, 0.29 \( \mu \)M, 0.31 \( \mu \)M, 3.6 \( \mu \)M and 47 \( \mu \)M, respectively. In addition, ONO-3307 inhibited both elastase release from N-formyl-Met-Leu-Phe (fMLP)-stimulated leukocytes and tissue thromboplastin release from endotoxin-stimulated leukocytes. To examine the effects of ONO-3307 on disseminated intravascular coagulation (DIC), we developed an experimental thrombosis model. ONO-3307 (10 mg/kg/hr) completely inhibited the deposition of radioactive fibrin in kidney and lung. Gabexate mesilate (50 mg/kg/hr) was also effective in this model, but the effect of nafamostat mesilate was unclear. These results indicate that ONO-3307 exhibits a wide range of inhibitory effects on various proteases, and ONO-3307 may be useful for the treatment of protease-mediated diseases such as thrombosis and DIC.

Proteases are known to play various important roles in maintaining physiological functions, and they involved in the pathogenesis of various diseases. DIC is a pathological syndrome in which the formation of a fibrin thrombus, the consumption of plasma coagulation factors and the activation of the fibrinolytic system are most likely to be attributed to the presence of thrombin in the systemic circulation (1). Antithrombin-III (AT-III) is the primary anti-coagulatory protein in circulating blood, and its anti-coagulant activity is accelerated by heparin through the formation of heparin-AT-III complex. Recently, it has been reported that the anti-coagulant activity of heparin-AT-III complex is neutralized by leukocyte elastase (2). However, the anti-coagulant activity of gabexate mesilate, a synthetic protease inhibitor, is not influenced by the presence of AT-III, and this compound has already been demonstrated to be effective for the treatment of DIC (3, 4). In the present study, inhibitory effects of ONO-3307 (4-sulfamoyl phenyl-4-guanidinobenzoate methanesulfonate) on various serine proteases and release of elastase and tissue thromboplastin from human leukocytes were investigated in vitro. Furthermore, we examined whether ONO-3307 has an inhibitory effect on experimental thrombosis induced by thrombin in vivo.

Materials and Methods

1) Enzymes and reagents: Bovine trypsin, human thrombin, human plasma kallikrein, bovine chymotrypsin, \( \alpha \)-N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA), succinyl-Ala-Pro-Ala-p-nitroanilide (succ-Ala-Pro-Ala-pNa), N-formyl-Met-Leu-Phe (fMLP) and cytochalasin B were purchased from Sigma Chemical Co., Ltd. (St. Louis, U.S.A.). Human plasmin was from Kabi Diagnostica (Stockholm, Sweden). Porcine pancreatic kallikrein and human leukocyte elastase were prepared in our laboratories. Bovine thrombin was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). S-2222, S-2238, S-2251, S-2266, S-2302 and
S-4511 as synthetic substrates and trans-1-amino methylcyclohexane carboxylic acid (t-AMCHA) were products of Daiichikagaku Pharmaceutical Co., Ltd. (Tokyo, Japan). Endotoxin (Escherichia coli O55:B5 lipopolysaccharide B) was obtained from Difco Lab. (Detroit, U.S.A.). The specific radioactivity of iodine-labelled human fibrinogen (\(^{125}\)I-fibrinogen; Life Science Laboratory Co., Ltd., Chiba, Japan) was 0.1 to 1.0 mCi/mg protein and contained more than 85% clottable protein. ONO-3307, gabexate mesilate and nafamostat mesilate were products of Ono Pharmaceutical Co., Ltd. (Osaka, Japan). The structural formula of ONO-3307 is shown in Fig. 1.

2) Determination of protease inhibitory activity: The hydrolytic activity of protease was determined using a synthetic substrate by the spectrophotometric method. The determination of the inhibitory activities on thrombin, plasmin, plasma kallikrein and pancreatic kallikrein and kinetic studies were performed by the method described by Ohno et al. (3). The inhibitory activities on trypsin and chymotrypsin were determined with BAPNA and S-4511 as synthetic substrates, respectively (5). The reaction was initiated by the addition of enzyme and carried out at 37°C. The enzyme activity was measured by the absorbance at 405 nm. Kinetic values were obtained in the absence and the presence of protease inhibitor by Lineweaver-Burk plots (6). The inhibitory activity on leukocyte elastase was determined by the method described by Levine et al. (7). Suc-Ala-Pro-Ala-pNa (0.1 mM) was incubated in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.2 M sodium chloride, and 0.001 unit of the enzyme in a final volume of 1 ml. After incubation at 37°C for 30 min, the absorbance was measured at 405 nm.

3) Effects of dialysis on thrombin inhibition and trypsin inhibition by ONO-3307: The reversibility of ONO-3307 on trypsin and thrombin was investigated by recovery of the protease activity on dialysis. Human plasma thrombin (2.5 U/ml) or bovine pancreatic trypsin (5 \(\mu\)g/ml) was incubated at 37°C for 5 min with ONO-3307, gabexate mesilate and nafamostat mesilate in phosphate-buffered saline, pH 7.4 (PBS) containing 1% BSA. The mixture was dialyzed using seamless cellulose tubing (Sanko Pharmaceutical Co., Ltd., Osaka, Japan) for 24 hr at room temperature against PBS in order to eliminate free forms of these synthetic protease inhibitors. After dialysis, thrombin activity was measured with 0.2 mM S-2238 in 25 mM Tris-HCl buffer, pH 8.3, containing 0.15 M NaCl, and trypsin activity was measured with 0.5 mM BAPNA in 20 mM HEPES-NaOH buffer, pH 8.0.

4) Effects on release of elastase from fMLP-stimulated human leukocytes: Human leukocytes were prepared by the method of Boyum (8). Human leukocytes (3.4 \(\times\) 10^6 cells/ml) were resuspended with Hanks solution, added to ONO-3307, cytochalasin B (5 \(\mu\)g/ml) and fMLP (0.04 \(\mu\)g/ml) before incubation at 37°C for 30 min. After incubation, the reaction mixture was centrifuged at 3000 r.p.m. at 4°C for 5 min, and elastase activity of the supernatant (the conditioned medium) was measured with suc-Ala-Pro-Ala-pNa as a synthetic substrate. The reaction mixture was composed of the conditioned medium (20 \(\mu\)l), 50 \(\mu\)mol of Tris-HCl buffer (pH 8.0), 0.5 \(\mu\)mol of suc-Ala-Pro-Ala-pNa and 100 \(\mu\)mol of NaCl in a final volume of 0.5 ml. Lactate dehydrogenase (LDH) activity of the conditioned medium was measured by the method described by Vassault (9).

5) Effects on release of tissue thromboplastin from endotoxin-stimulated human leukocytes: Effects on release of tissue thromboplastin by endotoxin were examined by the method of Inaba et al. (10). Human leukocytes were resuspended with Dulbecco minimum essential medium, added with ONO-3307 and endotoxin (10 \(\mu\)g/ml), and then incubated at 37°C for 4 hr. The reaction mix-

![Fig. 1. Structural formula of ONO-3307 (4-sulfamoyl phenyl-4-guanidinobenzoate methanesulfonate).](image-url)
ture was centrifuged at 1100 r.p.m., 4°C for 5 min, and tissue thromboplastin activity of the supernatant was measured with S-2222 as the substrate.

6) Effects on experimental thrombosis model in vivo: Effects on an experimental thrombosis model in vivo were examined by the method of Ohno et al. (12). Male Wistar rats weighing 180–220 g were anesthetized with ethyl carbamate (1.25 g/kg, i.p.). Both femoral veins were cannulated with Silastic tubing®, and the left kidney was surgically exposed without compromising vascular supply and drainage. A small gamma detector (Model PSM-321 Aloka) was placed just above the exposed kidney. A solution of $^{125}$I-fibrinogen (5 µCi) was infused; and subsequently, t-AMCHA (200 mg/kg) was injected through one of the catheters to prevent endogenous activation of fibrinolysis. ONO-3307 was continuously infused at a constant rate by an infusion pump. The changes of radioactivity in the kidney were continuously monitored and recorded. After infusion of ONO-3307, the rats were bled to death, and their organs were removed for the determination of radioactivity.

Results

1) Inhibitory effects on various proteases and its reversibility: Inhibitory effects of ONO-3307 on various proteases were examined using synthetic substrates in comparison with gabexate mesilate and nafamostat mesilate. As shown in Table 1, ONO-3307 inhibited trypsin, thrombin, plasma kallikrein, plasmin, pancreatic kallikrein and chymotrypsin, and their $K_i$ values were 0.048 µM, 0.18 µM, 0.29 µM, 0.31 µM, 3.6 µM and 47 µM, respectively. However, ONO-3307 had no inhibitory effect on leukocyte elastase up to the concentration of 1 mM. Gabexate mesilate and nafamostat mesilate also inhibited various proteases, and nafamostat mesilate had no inhibitory effect on leukocyte elastase up to the concentration of 1 mM. As for thrombin, plasmin, plasma kallikrein, pancreatic kallikrein and chymotrypsin, the inhibitory effect of gabexate mesilate was 10-fold less potent than that of ONO-3307. The manner of inhibition was competitive on these proteases (Fig. 2). These results indicated that ONO-3307 had a high affinity against thrombin, trypsin and plasma kallikrein. Furthermore, gabexate mesilate had a high affinity against trypsin, whereas nafamostat mesilate showed a high affinity against trypsin, plasmin and plasma kallikrein. With regards to inhibition of thrombin and plasmin, the $K_i$ ratio ($K_i$ value for plasmin/$K_i$ value for thrombin) of ONO-3307 was 1.7, a value which approximates that of gabexate mesilate (1.3). Namely ONO-3307 had a higher affinity on thrombin than plasmin. However, the ratio of nafamostat mesilate was 0.16 and is lower than that of gabexate mesilate. Nafamostat mesilate had a higher affinity on plasmin than thrombin.

The data presented in Table 2 show the reversibility of the inhibitory effect of ONO-3307, gabexate mesilate and nafamostat mesilate on thrombin and trypsin. ONO-3307 inhibited thrombin and trypsin; however, the effects disappeared on dialysis. ONO-3307 was found to bind trypsin and thrombin.

**Table 1. $K_i$ values of ONO-3307, gabexate mesilate and nafamostat mesilate on various proteases**

| Enzyme | Source          | Substrate | ONO-3307 $K_i$ (µM) | Gabexate mesilate $K_i$ (µM) | Nafamostat mesilate $K_i$ (µM) |
|--------|----------------|-----------|---------------------|-----------------------------|-------------------------------|
| Trypsin| Bovine pancreas| BAPNA     | 0.048               | 0.066                       | 0.084                         |
| Thrombin| Human plasma    | S-2238   | 0.18                | 1.5                         | 1.9                           |
| Plasmin| Human plasma    | S-2251   | 0.31                | 1.9                         | 0.31                          |
| Kallikrein| Porcine plasma | S-2266 | 3.6                 | 190                         | 0.79                          |
| Kallikrein| Human plasma    | S-2302   | 0.29                | 3.6                         | 0.042                         |
| Chymotrypsin| Bovine pancreas | S-4511 | 47                  | 160                         | 7.9                           |
| Elastase| Human leukocyte| Suc-APApNa | N.E.                | 160                         | N.E.                         |

$K_i$ values were obtained in the absence and presence of protease inhibitor by Lineweaver-Burk plots.  
$^{a}$Suc-Ala-Pro-Ala-pNa.  
$^{b}$No effect.
Fig. 2. Inhibitory manner of ONO-3307 for various proteases. Inhibition kinetics were studied by Lineweaver-Burk plots. The enzyme activity was measured in the absence (---) and the presence (●●●) of ONO-3307.

Table 2. Effects of dialysis on thrombin inhibition and trypsin inhibition by ONO-3307, nafamostat mesilate or gabexate mesilate

|                  | ONO-3307   | Gabexate mesilate | Nafamostat mesilate |
|------------------|------------|-------------------|---------------------|
|                  | 1 µM       | 5 µM              | 20 µM               | 100 µM              | 2 µM      | 10 µM     |
| Before dialysis  | 79.3       | 85.1              | 77.0                | 86.2                | 54.0      | 67.8      |
| After dialysis  | 0.0        | 0.0               | 0.0                 | 0.0                 | 0.0       | 0.0       |

b)

|                  | ONO-3307   | Gabexate mesilate | Nafamostat mesilate |
|------------------|------------|-------------------|---------------------|
|                  | 0.2 µM     | 1 µM              | 20 µM               | 0.2 µM              | 1 µM      |
| Before dialysis  | 23.3       | 100.0             | 87.7                | 24.7                | 100.0     |
| After dialysis  | 3.4        | 11.0              | 0.0                 | 1.3                 | 8.9       |

a) Human thrombin (2.5 u/ml) was incubated at 37°C for 5 min with ONO-3307 (1 µM or 5 µM), gabexate mesilate (20 µM or 100 µM) or nafamostat mesilate (2 µM or 10 µM) in PBS containing 1% BSA. The mixture was dialyzed against PBS at room temperature for 24 hr, and thrombin activity was measured with a synthetic substrate. b) Bovine trypsin (50 u/ml) was incubated at 37°C for 5 min with ONO-3307 (0.2 µM or 1 µM), gabexate mesilate (20 µM) or nafamostat mesilate (0.2 µM or 1 µM) in PBS containing 1% BSA. The mixture was dialyzed against PBS at room temperature for 24 hr, and trypsin activity was measured with a synthetic substrate.
reversely like gabexate mesilate and nafamostat mesilate.

2) Effects on release of elastase from fMLP-stimulated human leukocytes: Addition of cytochalasin B and fMLP induced release of elastase without the release of lactate dehydrogenase from human leukocytes (Fig. 3). Elastase activity indicated release as 30% of the total leukocyte elastase activity, whereas lactate dehydrogenase, as a cytosolic enzyme, was negligibly detected in the conditioned medium. Interestingly, ONO-3307 significantly inhibited the elastase activity of the conditioned medium, when 100 μM of ONO-3307 was incubated with human leukocytes, fMLP and cytochalasin B at 37°C for 30 min. On the other hand, gabexate mesilate and nafamostat mesilate (up to the concentration of 100 μM) had no inhibitory effects on the release of leukocyte elastase.

3) Effects on release of tissue thromboplastin from endotoxin-stimulated human leukocytes: Endotoxin induces release of tissue thromboplastin from human leukocytes (Fig. 4). ONO-3307 significantly inhibited tissue thromboplastin activity when 10 μM of ONO-3307 was incubated with human leukocytes and endotoxin at 37°C for 4 hr. The addition of gabexate mesilate and nafamostat mesilate also inhibited tissue thromboplastin activity of the supernatant. These results reveal that ONO-3307, gabexate mesilate and nafamostat mesilate inhibit the release of tissue thromboplastin or tissue thromboplastin activity.

4) Effects on experimental model in vivo: Infusion of thrombin after pretreatment of t-AMCHA produces experimental thrombosis in rats (12). A typical tracing pattern of radioactivity in the kidney is shown in Fig. 5. In the control group, radioactivity began to increase 15 min after the beginning of thrombin infusion and peaked 60 min after the completion of thrombin infusion. ONO-3307 (10 mg/kg/hr) completely inhibited the deposition of 125I-fibrinogen in the kidney. Similarly, gabex-

![Fig. 3](image-url) Inhibitory effects of ONO-3307, gabexate mesilate and nafamostat mesilate on the release of elastase from fMLP-stimulated leukocytes. Each column represents the mean±S.E. (n=4). N: Normal group, leukocytes were incubated without fMLP; C: Control group, leukocytes were incubated with fMLP; Protease inhibitor group: Leukocytes containing each concentration of protease inhibitor were incubated with fMLP. N.D.: not detectable. Significantly different from the control group: *P<0.05.
ate mesilate inhibited the deposition of $^{125}$I-fibrin in the kidney, but its effect was less potent than that of ONO-3307. However, nafamostat mesilate did not elicit a clear-cut effect up to 10 mg/kg/hr. We observed the death of rats when nafamostat mesilate (50 mg/mg/hr) was infused in this model. Effects of ONO-3307 on the deposition of $^{125}$I-fibrin in various tissues are shown in Fig. 6. In the control group, significant increases of the deposition of $^{125}$I-fibrin were seen in the kidney and lung. ONO-3307 inhibited the deposition of $^{125}$I-fibrin in these tissues. Though gabexate mesilate elicited a similar effect to ONO-3307, nafamostat mesilate had little effect.

**Discussion**

In the present study, inhibitory effects of ONO-3307 on various proteases were studied in comparison with gabexate mesilate and nafamostat mesilate in vitro. ONO-3307 competitively inhibited various proteases such as thrombin, trypsin and plasma kallikrein without any cofactor. The affinity of ONO-3307 for trypsin was slightly higher than that of gabexate mesilate and nafamostat mesilate. However, the affinity of ONO-3307 for human thrombin was about 10 times higher than that of gabexate mesilate or nafamostat mesilate.

Since gabexate mesilate and nafamostat mesilate are effective on DIC and acute pancreatitis as a thrombin inhibitor and a trypsin inhibitor, respectively (12, 13), ONO-3307 therefore can be conceived to elicit significant inhibitory effects on thrombin and trypsin.

On the inhibition of thrombin and plasmin, ONO-3307 showed a higher affinity on thrombin than plasmin, a finding that was similar to gabexate mesilate, corresponding closely to the $K_i$ values of ONO-3307 for human thrombin (0.18 $\mu M$) and plasmin (0.31 $\mu M$). However, nafamostat mesilate has a higher affinity on plasmin than thrombin, because the $K_i$ values were 1.9 $\mu M$ and 0.31 $\mu M$ for human thrombin and plasmin, respectively. The differences in affinity for these proteases may indicate that ONO-3307 and gabexate mesilate act as anti-coagulants and nafamostat mesilate acts as an anti-fibrinolysis agent in the blood.

It is known that heparin binds to AT-III in the blood, and this complex irreversibly inhibits thrombin (14). It is also reported that the plasma AT-III level is comparatively low in patients with DIC (15), and heparin is not effective on venous thrombosis in rats with low plasma AT-III level (16). We consider that the inhibitory effect of ONO-3307 is
Fig. 5. Effects of ONO-3307, gabexate mesilate and nafamostat mesilate on the deposition of \( ^{125}\text{I}-\)fibrin in the left kidneys of rats infused with thrombin.

Inhibitory effects of ONO-3307 on the experimental thrombosis model in vivo were studied in comparison with gabexate mesilate and nafamostat mesilate. ONO-3307 completely inhibited the formation of fibrin by the infusion of thrombin after pretreatment of t-AMCHA in vivo. This effect was 5–10 times more potent than that of gabexate mesilate. Although the inhibitory effect of nafamostat mesilate on thrombin in vitro was the same as that of gabexate mesilate, the antithrombotic effect of nafamostat mesilate was not clear in the experimental thrombosis model in vivo. The formation of thrombus induced by i.v. infusion of thrombin was markedly potentiated by t-AMCHA, which is an inhibitor of the fibrinolytic system (12). Nafamostat mesilate has a higher affinity on plasmin than thrombin; and the \( K_i \) values were 0.31 \( \mu \text{M} \) and 1.9 \( \mu \text{M} \) for human plasmin and thrombin, respectively. These results are probably due to the different affinity of each protease inhibitor against thrombin and plasmin. Our findings suggest that the formation of thrombi is important in the balance between the blood coagulation and fibrinolytic system of the circulating blood. ONO-3307 showed a higher affinity for thrombin than plasmin, and it elicited inhibitory effects on elastase and tissue thromboplastin. These reveal that ONO-3307 has a potential and beneficial use in the treatment of thrombotic disorders.
Fig. 6. Effects of ONO-3307, gabexate mesilate and nafamostat mesilate on the deposition of $^{125}$I-fibrin in various organs 120 min after thrombin infusion. Each column represents the mean±S.E. Normal group, n=4; Control group, n=6; Protease inhibitor group, n=6. Significantly different from the control group: *P<0.05, **P<0.01, ***P<0.001.

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