Pharmacological Studies of FUT-175, Nafamstat Mesilate
I. Inhibition of Protease Activity in in Vitro and in Vivo Experiments

Takuo AOYAMA, Yoshitaka INO, Masayuki OZEKI, Minoru ODA, Takuo SATO, Yoshiko KOSHIYAMA, Shoshi SUZUKI and Mitsunobu FUJITA

Research Laboratories, Torii & Co., Ltd., 3-14-3 Minamiyawata, Ichikawa, Chiba 272, Japan

Accepted March 26, 1984

Abstract—FUT-175, 6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate (nafamstat mesilate), a novel synthetic protease-inhibiting agent, was studied to determine its in vitro effects against various proteases and other enzymes, as well as to determine its in vivo protease inhibitory effects. FUT-175 was found to inhibit, in an intense, specific and reversible way, the enzyme activities of trypsin, C1r, C1s, thrombin, kallikrein and plasmin with IC50 values of the order of 10^{-6} - 10^{-8} M. FUT-175 also inhibited complement-mediated hemolysis, including both classical and alternative pathways, sites of inhibition being on C1r and C1s as evidenced by the intermediate-cell technique. In animal model reactions in which the complement system is known to be involved as pathogenetic factors, e.g., Forssman shock, Forssman cutaneous vasculitis, zymosan-induced paw edema, endotoxin shock and local Shwartzman reaction, FUT-175 was highly effective in that, for example, intravenous dosing at 3 mg/kg could completely protect guinea pigs from the lethal Forssman shock. FUT-175 was also found to be effective in trypsin-induced shock in mice, in lethality due to thrombin-thrombosis in mice and in kinin formation in the inflammatory process in rats.

Proteases are known to play various important roles in maintaining normal physiological function, for example, thrombin and plasmin in the blood coagulation and fibrinolysis system and the complements in the host defense against infections. Proteases, however, are also known to be involved in the pathogenesis of various diseases, for example, trypsin and kallikrein in the pancreatitis, thrombin and plasmin in the disseminated intravascular coagulation syndroms, and the complement system in the autoimmune diseases such as systemic lupus erythematosus, and acute pancreatitis. In-

Fig. 1. Structural formula of FUT-175 (nafamstat mesilate)
hibition of anomalously activated enzymes in diseases, thus, would provide a useful therapeutic means.

FUT-175, 6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate (nafamstat mesilate), is a novel synthetic protease-inhibiting agent and has the chemical structure shown in Fig. 1. It has been developed by Torii & Co., Ltd., and its basic biochemical and pharmacological properties have already been published by Fujii and Hitomi (1, 2).

In the present report, FUT-175 was studied to determine its in vitro effects against various proteases and other enzymes as well as to determine its in vivo protease inhibitory effects. This paper consists of five parts: I. Inhibitory effects on enzymes, II. Effects on the complement system, III. Effects on trypsin, IV. Effects on thrombin, and V. Effects on the kinin formation system.

Materials and Methods

I. Inhibitory effects on enzymes

C1r and C1s were obtained and purified from preserved human serum as described by Okamura et al. (3, 4). Other enzymes used were trypsin (Sigma), pancreatic kallikrein (Bayer), papain (Sigma), pepsin (Sigma), cathepsin D (Sigma), thrombin (Mochida Pharm.), plasmin (Green Cross Corp.), chymotrypsin (Biochemicals, Inc.), elastase (Koch-Light), collagenase (Biochemicals, Inc.), phospholipase A2 (Boehringer Mannheim Yamanouchi, Inc.) and pancreatic lipase (Sigma). Substrates used were tosyl-L-arginine methyl ester (TAME), acetyl-glycyl-L-lysine methylester (AGLM), acetyl-L-arginine methylester (AAME), benzoyl-L-arginine ethylester (BAEE), succinyl-L-alanyl-L-prolyl-L-alanine 4-methylcoumaryl-7-amide (SucAlaProAlaMCA), carbozonyx-glycyl-L-prolyl-L-leucyl-glycyl-L-proline (ZGlyProLeuGlyPro) and 2,3-dimercaptoopropan-1-ol tributyrate (BALB) from the Protein Research Foundation and a-casein, hemoglobin and phosphatidyl choline from Sigma. Other materials used were a2-macroglobulin (a2MG, BMY), aprotinin (Trasylol®, Bayer), soybean trypsin inhibitor (SBTI, Sigma) and the lipase assay set (Lipase Kit S®, Dainippon Pharm.)

1. Determination of enzyme inhibitory activity
1) Proteases

i) C1s, C1f, thrombin, pancreatic kallikrein, plasmin and trypsin (synthetic substrates): Assay was done by the method of Hestrin as modified by Roberts (5). The substrates used were AGLME for C1s, AAME for C1f, and TAME for thrombin, pancreatic kallikrein, plasmin and trypsin, at a substrate concentration of 10 mM. Hydrolytic activities of each protease on the substrate, either in the presence or absence of FUT-175, were determined by incubation of reaction mixtures in 100 mM borate buffer (pH 8.5) for pancreatic kallikrein and plasmin, 10 mM CaCl2-containing 100 mM borate buffer (pH 8.5) for trypsin, 100 mM phosphate buffer (pH 7.4) for C1s and C1f, and 20 mM phosphate buffer (pH 7.4) for thrombin, at 37°C for 30 min.

ii) Chymotrypsin, trypsin and plasmin (casein): The method described by Mura-matsu et al. (6) was followed.

iii) Papain (BAEE): The reaction mixtures consisting of 0.35 U/ml papain, FUT-175 at varying concentrations, 50 mM BAEE, 5 mM cysteine and 1 mM EDTA-2Na as an activator in 50 mM Tris-HCl buffer (pH 7.0) were incubated at 37°C for 30 min, and the unhydrolyzed BAEE was quantitated by Hestrin’s method (op. cit.).

iv) Pepsin and cathepsin D (hemoglobin): These assays were done by the method described by Cunningham et al. (7).

v) Elastase (SucAlaProAlaMCA): The assay was done by the method described by Monta et al. (8).

vi) Collagenase (ZGlyProLeuGlyPro): The method of Yagisawa et al. (9) was modified. The reaction mixtures consisting of 5 U/ml collagenase, FUT-175 at varying concentrations, and 1 mM of the substrate in 1 mM CaCl2-containing 20 mM Veronal-HCl buffer (pH 8.2) were incubated at 37°C for 30 min. After incubation, EDTA-2Na, Fluorescamin in acetone and 30% acetic acid were added, and the fluorescence was quantitated at Ex 390 nm and Em 475 nm.

2) Other enzymes

i) Lipase (BALB): The assay was done using Lipase Kit S®, according to the method
Pharmacological Studies of FUT-175

of Kurooka and Kitamura (10).

ii) Phospholipase A2 (phosphatidyl choline): The method described by de Haas et al. (11) was followed.

2. Studies on the inhibition mechanism

1) Kinetic study—determination of Ki value

The proteases studied were C1r, C1s, trypsin, thrombin, kallikrein and plasmin. The rates of hydrolysis of each corresponding substrate by the action of each protease were determined in the presence of FUT-175 using Hestrin’s method. Inhibition kinetics were studied by Lineweaver-Burk’s plot method (12), and Ki value was obtained graphically by Dixon plots (13).

2) Reversibility

The proteases studied were trypsin and thrombin. The reversibility of FUT-175 protease inhibition was investigated by observing the recovery of the esterolytic activity of the FUT-175-treated enzyme by dialysis.

Dialysis was performed against the buffer at 4°C overnight; and immediately after finishing the dialysis and 1, 2, 3, 4 and 5 hr after incubation at 37°C, esterolytic activity of trypsin and thrombin on TAME was determined by Hestrin’s method.

3) Effect on α2MG-trypsin complex

The buffer solution used was 10 mM CaCl2-containing 100 mM borate buffer, pH 8.5. Trypsin and α2MG (1:10 in weight) were incubated at 37°C for 15 min to form the trypsin-α2MG complex. Inhibition of trypsin activity by FUT-175 was determined by Hestrin’s method, using 12.5 mM TAME, with incubation at 37°C for 30 min in the presence of 2 μg/ml SBTI.

II. Effects on the complement system

C1 and C2 were obtained and purified from guinea pig serum according to the method described by Nishioka et al. (14). Other chemicals and drugs used were sheep erythrocytes (SRBC, Pharm.), rabbit anti-SRBC serum (hemolysin, Kyokuto Chemicals, Inc.), goat anti-guinea pig C3 serum (Cappel Labs., Inc.), endotoxin (LPS B E. coli 055B5 and LPS W E. coli 0127:B12, Difco), platelastin plus activator (General Diagnostics), leupeptin (Protein Research Foundation), zymosan (Sigma), gabexate mesilate (FOY®, Ono Pharm.), indomethacin (IDM, Indacin®, Nippon Merck Banyu), hydrocortisone (Wako Pure Chemicals) and EDTA-2Na (Wako Pure Chemicals). Animals used were mice (male ICR, weighing 22-24 g; Japan Charles River), rats (male Sprague-Dawley, weighing 200-250 g; Japan Charles River) and guinea pigs (male Hartley, weighing 250-300 g; Matsumoto Experimental Animals).

1. Complement-mediated hemolysis

1) In vitro experiments

i) Classical pathway:

i)-a. Experiments with diluted serum: The method of Mayer (15) was followed. The mixtures consisting of EA, sheep erythrocytes sensitized beforehand with 500-fold diluted hemolysin, at a final concentration of 2.5 x 10² cells/ml, 1/2.5 vol. of 200-fold diluted guinea pig serum and FUT-175 at varying concentrations in GVB++ buffer were incubated at 37°C for 10 min. After incubation, the reaction was stopped by adding EDTA-containing saline, the mixture was centrifuged at 3000 r.p.m. for 10 min, and the absorbance of the supernatant was read at 540 nm.

The effect of FUT-175 on the complement concentration-hemolysis relationship was studied by the same procedure, except guinea pig serum in varying dilutions of 1:50–1:400 was used instead of the given dilution of 1:200.

i)-b. Experiments with undiluted serum: The experiments were conducted by the method of Plescia et al. (16), with modifications. Mixtures were prepared on ice by mixing 1.5 ml of undiluted guinea pig serum, 1.2 ml of 5 x 10⁸ cells/ml EA and 0.3 ml of either FUT-175 solution or the buffer. After thorough mixing, they were transferred to an incubator at 37°C, and 0.1-ml aliquots were withdrawn at 15 sec intervals for hemolysis determination. Hemolysis was determined by adding 1.9 ml of EDTA-containing saline to a 0.1-ml aliquot and reading the absorbance at 540 nm of the supernatant obtained by centrifugation at 3000 r.p.m. for 10 min.

i)-c. Sites of FUT-175 inhibition on early components in the complement-mediated hemolysis: The complement-mediated hemolysis via a classical pathway activation
was reconstituted by means of an intermediate cell technique, namely, EAC4 was converted to EAC14, and finally the hemolysis of EA was provoked by adding C3–C9 as the C-EDTA (the guinea pig serum diluted by 12.5-fold with 0.04 M EDTA-GVB) to pre-formed EAC142.

Procedures described by Nishioka et al. (op. cit.) were followed, and FUT-175 was added to each incubation mixture of EAC4 and C1, EAC14 and C2, and of EAC142 and C-EDTA to study the FUT-175 inhibition on C1, C2 and any of the late components, respectively.

ii) Alternative pathway: Determinations were carried out by a modification of the methods described by Platts-Mills and Ishizaka (17).

The mixtures of unsensitized mouse erythrocytes at a final concentration of 8x10^7 cells/ml, 1/5 vol. of normal human plasma in a dilution of 1:2 and FUT-175 at varying concentrations in EGTA-GVB buffer were incubated at 37°C for 10 min. After addition of EDTA-containing saline, the mixture was centrifuged at 3000 r.p.m. for 10 min, and the absorbance of the supernatant was read at 414 nm.

2) In vivo experiments

i) Classical pathway: Groups of 5–6 guinea pigs were used. Three min after intravenous injection of 1 ml of 10^10 cells/ml sensitized SRBC, 0.5 ml of blood was withdrawn by cardiac puncture. After addition of 4.5 ml EDTA-saline, the supernatant was obtained by centrifugation at 2000 r.p.m. for 10 min, and the absorbance at 541 nm was recorded. Drugs were dosed 1 min for i.v. and 1 hr for p.o. administration, respectively, prior to the hemolysin injection.

ii) Alternative pathway: Groups of 5 guinea pigs were used. Guinea pigs received i.v. injection of 2.5 ml/kg of dog serum, and 0.3 ml of blood was obtained 15 min later by cardiac puncture. To this was added 2.7 ml of 10 mM EDTA in saline, then it was centrifuged at 2000 r.p.m. for 10 min, and the absorbance of the supernatant was read at 541 nm. Drugs were dosed 1 min for i.v. and 1 hr for p.o. administration, respectively, before i.v. injection of dog serum.

2. Forssman reaction

1) Systemic Forssman shock

i) Determination of hemolysin dose: Forssman shock was provoked as described by Glovsky et al. (18). To determine the appropriate dose of hemolysin, varying doses of 0.1–0.5 ml/animal were injected intravenously to guinea pigs. Survival time after hemolysin injection was recorded, and the weight of the lung taken immediately after death was also recorded. Guinea pigs surviving a period of 10 min after hemolysin injection were sacrificed by exsanguination, and the weight of the lung was also recorded.

ii) Survival time and lung weight in Forssman shock: Groups of 6–10 guinea pigs were used. To observe the survival time, hemolysin at a dose of 0.5 ml/animal was intravenously injected. For lung weight determination, 0.2 ml/animal of hemolysin was intravenously injected, and the guinea pigs were sacrificed 10 min later. Drugs were dosed 5 min for i.v. and 1 hr for p.o. administration, respectively, prior to the hemolysin injection.

iii) Effects on parameter changes: The effects of FUT-175 on the Forssman reaction-induced changes in CHSO, C3 level, C4 level, erythrocyte count, leucocyte count, platelet count and blood coagulation time were studied in guinea pigs in groups of 5–8. Hemolysin at a dose of 0.5 ml/animal was intravenously injected, and the blood specimens were taken 5 min later. The drugs were dosed intravenously 5 min before hemolysin injection.

Hemolytic complement in whole serum was determined as CH50 by the method of Mayer (op. cit.), the erythrocyte and leucocyte counts on the Coulter counter and the platelet count on the platelet counter (PL-100, Sysmex). Blood coagulation time was determined as APTT using platelet plus activator. Serum C3 level was determined as the C3 protein amount by means of single radial immunodiffusion using goat anti-guinea pig C3 serum. Serum C4 level was determined as the hemolytic activity by means of an intermediate cell technique as described by Kitamura (19). Effects of drugs on C3 and C4 levels were evaluated as a percentage of the control level.
2) Forssman cutaneous vasculitis

Into two sites located laterally in the shaved back skin of guinea pigs, in groups of 5 each, was intradermally injected 0.1 ml each of hemolysin diluted to 1/2 concentration with saline; and 3 hr later, the degree of reaction was given one of five grades: 0, no detectable changes; 1, edema and/or mild erythema; 2, moderate erythema; 3, definite hemorrhage and 4, necrosis of dark reddish appearance in the center. Drugs were dosed intraperitoneally 30 min before challenge.

3. Zymosan-induced paw edema

Groups of 5-7 rats were used. Into the hind footpad was subcutaneously injected 0.1 ml of a 6% suspension of pre-washed zymosan in 1% CMC, and volumes of the footpad in both inflamed and non-inflamed states were measured at various times after challenge to calculate the percent edema formation. Drugs were intravenously administered 1 min before challenge.

At each determination, the percent inhibition of edema formation against the control group was calculated based on comparison of the area under the curve, and either the ED50 or ED25 value for each compound was obtained by the method of Litchfield-Wilcoxon (20).

4. Endotoxin shock

Groups of 20 mice were used. Endotoxin was injected intraperitoneally, and the survival rate was evaluated at 24 hr and 48 hr after endotoxin injection. Drugs were dosed intravenously and intraperitoneally 1 min prior to endotoxin injection.

5. Local Shwartzman reaction

Groups of 5 guinea pigs were used. Into the shaved back skin was intradermally injected endotoxin at a dose of 100 μg/0.1 ml/site, and the reaction was induced 24 hr later by an intravenous injection of endotoxin of 1 mg/ml/animal. The degree of the reaction was recorded at 3, 5 and 24 hr after challenge and assigned one of five grades: 0, practically no changes; 1, mild erythema; 2, definite erythema; 3, erythema with small necrosis and 4, necrosis.

III. Effects on trypsin

Reagents used were trypsin (bovine pancreatic crystalline-Type III, Sigma), apro-}

thonin (Trasylol®, Bayer), gabexate mesilate (FOY®, Ono Pharm.) and t-butoxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methylcoumaryl-7-amide (BocPheSerArgMCA, Protein Research Foundation).

Animals used were mice (male ICR, weighing 25–28 g; Japan Charles River) and rabbits (male Japanese mongrel albino, weighing 3.0–3.5 kg; Matsumoto Experimental Animals and Takasugi Experimental Animals).

1. In vivo trypsin inhibitory effects

Rabbits anesthetized with i.v. Na pentobarbital at 35 mg/kg were intravenously infused for 1 hr (0.162 ml/min) with FUT-175 at varying doses and trypsin at 10 μg/kg/min simultaneously and separately through the right and left marginal ear veins, respectively, by means of an infusion pump (Harvard apparatus, Model 1210). Blood specimens, as 0.38% citrated blood (1:9), were taken immediately before and 15, 30, 45 and 60 min after start of the infusion, and plasma was separated by centrifugation at 3000 r.p.m. for 10 min and determined for trypsin activity.

Trypsin activity was determined by the following procedure: reaction mixtures consisting of 0.8 ml of 0.125 mM BocPheSer-ArgMCA-containing 50 mM Tris-HCl buffer (pH 7.4), 0.1 ml of the plasma and 0.1 ml of the buffer were incubated at 37°C for 15 min; to these were added 1.0 ml of the stopping solution of 30% acetic acid and then, 1.0 ml of distilled water; and the fluorescence of AMC released was read at Ex 380 nm and Em 460 nm on a spectrofluorophotometer (Hitachi, Model MPF-3).

2. Trypsin-induced shock

Mice in groups of 5–12 were used. After 1 min following i.v. dosing of drugs, trypsin was intravenously injected at a dose of 0.52 mg/animal, and the number of dead mice were counted 2 hr later.

IV. Effects on thrombin

Reagents used were thrombin (Mochida Pharm.), Data-Fi Fibrinogen Determination Reagents (Dade), heparin (Wako Pure Chemicals) and gabexate mesilate (FOY®, Ono Pharm.).

Animals used were mice (male ICR, weighing 24–28 g; Japan Charles River) and
rabbits (male Japanese mongrel albino, weighing 3.0–4.7 kg; Matsumoto Experimental Animals).

1. In vivo thrombin inhibitory effects

Rabbits anesthetized with i.v. Na pentobarbital at 35 mg/kg received intravenous infusion of thrombin at 2 U/kg/min via the jugular vein and FUT-175 at 1, 10 or 100 µg/kg/min via the ear marginal vein, simultaneously, for 1 hr (12.4 ml/hr). Blood specimens were taken via the carotid artery into a syringe containing 1/10 vol. of 3.8% Na citrate, and for fibrinogen determination, the plasma was immediately obtained by centrifugation at 3000 r.p.m. for 10 min. Plasma fibrinogen levels were determined by means of the thrombin time using Data-Fi Fibrinogen Determination Reagents. Groups of 4–5 rabbits were used.

2. Effects on thrombin-thrombosis

Groups of 10 mice were used. Mice each received an intravenous injection of 300 U/kg of thrombin, and the mortality was determined 1 and 24 hr later. FUT-175, at doses of 0.3, 1, 3 and 10 mg/kg, was dosed through the tail vein 1 min before thrombin injection.

V. Effects on the kinin formation system

Glandular kallikrein was prepared from rat submandibular glands. The glands were excised from rats under anesthesia with pentobarbital sodium, 35 mg/kg, i.p., and weighed. These were homogenized on ice in 3 vol. of 100 mM NaCl-containing 10 mM phosphate buffer (pH 7.4). To the supernatant obtained by centrifugation of the homogenate at 3000 r.p.m. for 10 min at 4°C was added solid (NH₄)₂SO₄ to 80% saturation, and the precipitate formed was collected, dissolved in the same buffer and dialyzed overnight against the same buffer.

After dialysis, fractionation was conducted on a DEAE-cellulose column with a linear gradient of 100 mM–500 mM NaCl in 10 mM phosphate buffer (pH 6.0). The fractions having TAME hydrolytic activity were collected and pooled.

The potency of a 1 ml aliquot of the pooled fractions used in the present experiments was found to be equivalent to 100 mU of porcine pancreatic kallikrein in terms of the blood flow increment after i.v. injection in dogs.

Other reagents used were porcine pancreatic kallikrein (Bayer), trypsin (Sigma), bradykinin (Protein Research Foundation), TAME (Protein Research Foundation), pentobarbital sodium (Dainippon Pharm.), dibenamine (Tokyo Chemicals), estradiol (Yamanouchi Pharm.), atropine sulfate (Iwaki) and o-phenanthroline (Wako Pure Chemicals).

Animals used were rats (male Sprague-Dawley, weighing 200–250 g; Japan Charles River) and mongrel dogs (male adults, weighing approx. 10 kg).

Assay of kinin formed was achieved by determining the uterus contraction-inducing potency in comparison with bradykinin, kinin activity being expressed as the bradykinin equivalents (ng BK eq/ml).

Female rats, each dosed with estradiol at 1 mg/kg intraperitoneally and subcutaneously 18–20 hr before, were killed by exsanguination, and their uteri were excised. The isolated uterus was mounted in a 10-ml organ bath containing low Ca Locke-Ringer’s solution at 23±1°C under aeration with 95% O₂–5% CO₂. Contractions were recorded on sooted paper by means of isotonic levers with 1 g load. Low Ca Locke-Ringer’s solution was of the following composition (mM): NaCl, 154; KCl, 5.6; CaCl₂, 1.1; MgCl₂, 2.1; NaHCO₃, 2.4 and glucose, 2.8, and it contained 1×10⁻⁶ M each of atropine sulfate and dibenamine.

1. Kinin formation in plasma

i) In vitro experiments: In vitro effects of FUT-175 on the kinin formation were studied on each rat and dog plasma. Kinin-generating substances used were each 100 mg/ml glass powder and 1 mg/ml trypsin for both rat and dog plasma and rat submandibular kallikrein preparation at 1:160 dilution for rat plasma.

The reaction mixtures containing 0.2 ml of FUT-175 at varying concentrations, 0.5 ml of plasma, 0.5 ml of kinin-generating substance, 0.4 ml of 2 mg/ml o-phenanthroline and 0.4 ml of 100 mM phosphate buffer (pH 7.4) in plastic tubes were incubated at 37°C for 10 min. After the reaction was stopped by the addition of 5 ml of ethanol at 70°C, the mixtures were kept at 70°C for a further 10 min and then centrifuged at 3000 r.p.m. for 15 min. The supernatants thus obtained were
dried in vacuo at approx. 40°C, and the residues were kept at −20°C before subjected to assay.

ii) Ex vivo experiments: The plasma specimens were obtained from rats in groups of 5–6 5 min after i.v. dosing of FUT-175, and processed by the same procedure, using glass powder as the kinin-generating substance, as described in the preceding section, including storage at −20°C before assay.

2. Kinin formation in the inflammatory process

Kinin formation at the sites of inflammation and effects of FUT-175 on it were studied in rats by means of heat (46°C)-induced inflammation as described by Garcia et al. (21), using the double coaxial perfusion technique. Perfusion was performed at a rate of 0.103 ml/min using a continuous perfusion pump (Harvard apparatus, Model 1210), and the pre-perfusates were obtained for 15 min from the rat paw at room temperature. Then, the rat paw was immersed in water of 46°C, and 3 portions of test-perfusates were collected at 15-min intervals. Activities in perfusates were assayed on the isolated rat uterus by comparison with a bradykinin standard. The perfusion fluid was Tyrode solution having the following composition (mM): NaCl, 136.9; KCl, 2.7; CaCl2, 1.8; MgCl2, 0.5; NaHCO3, 11.9; NaH2PO4, 0.4 and glucose, 5.6.

FUT-175 was dosed intravenously immediately before the paw was immersed in water of 46°C.

Results

I. Inhibitory effects on enzymes

1. Enzyme inhibitory activity

1) Proteases

Table 1 shows the inhibitory effectiveness of FUT-175 in terms of IC50 values, the concentration of FUT-175 that inhibits each protease activity by 50%.

As represented in Table 1, FUT-175 showed potent inhibition against C1r, C1s, trypsin, thrombin, kallikrein and plasmin with IC50s in the order of 10−6−10−8 M and potent inhibition was similarly observed on casein digestion by trypsin and plasmin. Against chymotrypsin and collagenase, however, the effectiveness of FUT-175 was moderate, with IC50s of 5.0×10−4 M and 4.2×10−4 M, respectively; and it had practically no inhibitory activity against pepsin and cathepsin D as well as elastase and papain with IC50s higher than 10−3 M.

2) Other enzymes

| Enzyme       | Substrate        | Inhibitor concentration (IC50: M) |
|--------------|------------------|-----------------------------------|
| Trypsin      | TAME             | 1.3×10−8                          |
|              | Casein           | 1.7×10−7                          |
| Thrombin     | TAME             | 1.9×10−8                          |
|              | Casein           | 1.2×10−7                          |
| Plasmin      | TAME             | 2.9×10−6                          |
|              | Casein           | 3.9×10−6                          |
| Kallikrein   | TAME             | 8.0×10−7                          |
| C1r          | AAME             | 2.9×10−8                          |
| C1s          | AGLME            | 5.0×10−4                          |
| Chymotrypsin | Casein           | >10−3                             |
| Elastase     | SucAlaProAlaMCA  | >10−3                             |
| Papain       | BAEE             | >10−3                             |
| Collagenase  | ZGlyProLeuGlyPro | 4.2×10−4                          |
| Pepsin       | Hemoglobin       | >10−3                             |
| Cathepsin D  | Hemoglobin       | >10−3                             |
| Phospholipase A2 | Phosphatidylcholine | 7.0×10−5                        |
| Lipase       | BALB             | 8.4×10−4                          |

Each value represents IC50 values (M), the concentration of FUT-175 to inhibit 50% of each enzyme activity.
The inhibitory effectiveness of FUT-175 was moderate against phospholipase A2 with an IC50 of $7.0 \times 10^{-5}$ M, and it was weak against lipase with an IC50 of $8.4 \times 10^{-4}$ M (Table 1).

2. The inhibition mechanism

1) Kinetic study—$K_i$ value

Figure 2 represents Lineweaver-Burk plots and $K_i$ values obtained for each of the proteases studied. As shown in Fig. 2, FUT-175 inhibited the esterolytic activity of C1r, thrombin, plasmin and kallikrein in a competitive manner and inhibited C1s and trypsin in a non-competitive manner. Affinity of FUT-175 to these proteases was shown to be high by $K_i$ values in the order of $10^{-7}$–$10^{-8}$ M.

2) Reversibility

After overnight dialysis of FUT-175-pretreated proteases at 4°C, recovery of the esterolytic activity was approx. 100% for thrombin, but only approx. 20% for trypsin, as shown in Fig. 3. The esterolytic activity of trypsin, however, was observed to be restored nearly to 100% in the assay done after 5 hr incubation at 37°C following overnight dialysis at 4°C.

3) Effect on the $\alpha_2$MG-trypsin complex

The effect was studied using a mixture of trypsin and $\alpha_2$MG, which had been incubated long enough to expect sufficient formation of the complex, in the presence of SBT1 to inhibit free trypsin. The study revealed that FUT-175, at a concentration of $1 \times 10^{-7}$ M, inhibited the esterolytic activity of trypsin by 69.8% for the $\alpha_2$MG-complexed enzyme and by 84.0% for the free enzyme. Aprotinin concomitantly studied was found, on the other hand, to exert inhibition of only 41.8% at a concentration of 500 KIU/ml.
Pharmacological Studies of FUT-175

against the \( \alpha_2 \)-MG-complexed enzyme, whereas 31.3 KIU/ml of aprotinin inhibited the free trypsin by 50.2\%, the effectiveness on the former being approx. 1/16 of that on the latter (Table 2).

II. Effects on the complement system

1. Complement-mediated hemolysis
   1) In vitro experiments
      i) Classical pathway:
         i)-a. Experiments with diluted serum: Results represented in Fig. 4 revealed that FUT-175 inhibited the complement-mediated hemolysis via classical pathway activation in diluted serum, in a concentration-dependent manner, with an IC50 of \( 3.0 \times 10^{-8} \) M. In Fig. 5 are shown the results of experiments using guinea pig sera of serial dilution to study the effect of FUT-175 on the complement concentration-hemolysis relationship. The results showed that the hemolysis of sensitized SRBC occurred depending on the complement concentration, and FUT-175 affected this relationship by increasing the complement concentration required to produce 50% hemolysis. In fact, although in the absence of FUT-175, 50% hemolysis was caused by serum dilution

| Inhibitor   | Concentration of inhibitor | Inhibition (%) |
|-------------|----------------------------|----------------|
|             |                            | Trypsin        | Trypsin-\( \alpha_2 \)-macroglobulin complex |
| FUT-175     | \( 1 \times 10^{-9} \) M   | 20.3           | 15.1 |
|             | \( 1 \times 10^{-7} \)     | 84.0           | 69.8 |
|             | \( 1 \times 10^{-6} \)     | 100            | 100 |
| Aprotinin   | 7.8 KIU                    | 13.2           | 0    |
|             | 31.3                       | 50.2           | 9.4  |
|             | 125                        | 86.1           | 22.4 |
|             | 500                        | 100            | 41.8 |

Each value represents percent inhibition of esterolytic activities of trypsin and \( \alpha_2 \)-macroglobulin-trypsin complex at indicated concentrations of FUT-175 and aprotinin.

Fig. 4. Inhibitory effect of FUT-175 on classical pathway-mediated hemolysis. Classical pathway-mediated hemolysis was achieved by mixing sensitized SRBC and 200-fold diluted guinea pig serum. Each point represents percent inhibition of the control hemolysis at each concentration of FUT-175.

Fig. 5. Effect of FUT-175 on the complement concentration-hemolysis relationship. Guinea pig sera in varying dilutions of 1:50–1:400 were used as the complement source. Sensitized SRBC was hemolyzed with guinea pig serum in the presence or absence of FUT-175 (\( 1 \times 10^{-5} \) M). Each point represents percent hemolysis of the control at each dilution of serum. Abscissa is plotted in logarithmic scale.
of approx. 1:225, it was caused by a serum dilution of 1:125 in the presence of $1.0 \times 10^7$ M FUT-175.

i)-b. Experiments with undiluted serum: Results obtained are depicted in Fig. 6. The hemolysis in the system using undiluted serum occurred progressively with time, with a T50 (time required to obtain 50% hemolysis) of 41 sec and a S (slope of the regression line calculated using the portion of the curve corresponding to 20% to 80% hemolysis) of 1.607. In the presence of $3.0 \times 10^6$ M FUT-175, however, the hemolysis was markedly suppressed, namely with a T50 of 135 sec and a S of 0.343. When sensitized sheep erythrocytes were added into the guinea pig serum preincubated with $3.0 \times 10^{-6}$ M FUT-175 at 37°C for 10 min, the hemolysis was also suppressed with a T50 of 113 sec and a S of 0.429.

i)-c. Sites of FUT-175 inhibition on early components in the complement-mediated hemolysis: In the complement-mediated hemolysis of the classical pathway system, FUT-175 was found to inhibit the hemolysis markedly and in a concentration-dependent manner when added to the incubation mixtures of EAC4 and C1, EAC14 and C2, and EAC142 and C3–9 (EDTA-treated guinea pig serum), were used. FUT-175 was added to incubation mixtures of EAC4 and C1, EAC14 and C2, and EAC142 and C3–9. Each point represents percent inhibition of hemolysis by FUT-175 at varying concentrations.

did not inhibit the hemolysis when added to the incubation mixtures of EAC142 and C-EDTA, containing C3–C9, and in fact, the inhibition observed was only 10.4% with $1.0 \times 10^{-3}$ M FUT-175 (Fig. 7).

ii) Alternative pathway: In the complement-
mediated hemolysis via an alternative pathway activation by means of the unsensitized mouse erythrocyte-human plasma system. FUT-175 was also inhibitory with an IC50 of $3.0 \times 10^{-7}$ M (Fig. 8).

2. In vivo experiments

i) Classical pathway: As shown in Table 3, FUT-175 significantly suppressed the in vivo hemolysis, the inhibition being 83.8% and 100% at doses of 0.3 and 1.0 mg/kg, i.v., respectively. After oral administration, a dose of 10 mg/kg was practically ineffective, but 23.8% and 54.4% of inhibition were obtained at doses of 30 and 100 mg/kg, respectively.

It was confirmed that no hemolysis occurred when unsensitized SRBC were injected intravenously to normal guinea pigs.

ii) Alternative pathway: Intravenous dosing of FUT-175 at doses of 0.3, 1.0 and 3.0 mg/kg suppressed the in vivo hemolysis reaction dose-dependently, but gabexate mesilate was found to be ineffective even at a dose of 10 mg/kg.

Oral dosing of FUT-175 at doses of 10, 30 and 100 mg/kg also inhibited the hemolysis.

![Fig. 8](image-url)  
**Fig. 8.** Inhibitory effect of FUT-175 on alternative pathway-mediated hemolysis. Alternative pathway-mediated hemolysis was performed by mixing mouse erythrocytes and 2-fold diluted human plasma. Each point represents percent inhibition of hemolysis of the control by FUT-175 at each concentration indicated.

| Dose (mg/kg) | Route | Inhibition of hemolysis (%) |
|--------------|-------|----------------------------|
| 0.1          | i.v.  | 36.2                       |
| 0.3          |       | 83.8                       |
| 1.0          |       | 100                        |
| 10           | p.o.  | 0.5                        |
| 30           |       | 23.8                       |
| 100          |       | 54.4                       |

Groups of 5–6 guinea pigs were used. Drugs were dosed 1 min for the intravenous route and 1 hr for the oral route before injection of sensitized-SRBC. Each value represents percent inhibition of the control.

| Drug             | Dose (mg/kg) | Route | Inhibition of hemolysis (%) |
|------------------|--------------|-------|----------------------------|
| FUT-175          | 0.3          | i.v.  | 50.4                       |
|                  | 1.0          |       | 76.7                       |
|                  | 3.0          |       | 97.7                       |
| Gabexate mesilate| 10           |       | 9.0                        |
| FUT-175          | 10           | p.o.  | 28.6                       |
|                  | 30           |       | 42.8                       |
|                  | 100          |       | 57.7                       |
| Gabexate mesilate| 100          |       | 30.0                       |

Groups of 5 guinea pigs were used. Drugs were each dosed 1 min for the intravenous route and 1 hr for the oral route before injection of dog serum.
in a dose-dependent manner, but the suppression by gabexate mesilate was only 30% at a dose of 100 mg/kg (Table 4).

In this alternative pathway-mediated in vivo hemolysis system, inactivated dog serum, at 56°C for 30 min, was confirmed to induce no hemolysis.

**2. Forssman reaction**

1) **Systemic Forssman shock**

i) **Determination of hemolysin dose:**

Hemolysin, at doses of 0.1 to 0.5 ml/animal, was found to increase both the mortality and lung weight dose-dependently, and in fact, mortalities of 0, 0, 13, 50 and 100% were recorded during a period of 10 min after hemolysin i.v. injection at doses of 0.1, 0.2, 0.3, 0.4 and 0.5 ml, respectively. Hemolysin was also shown to increase the lung weights dose-dependently, being mainly attributed to edema formation and hemorrhage, and more precisely, no increase at 0.1 ml, dose-dependent increases at 0.2–0.4 ml, and the increases caused by doses of 0.4 and 0.5 ml were maximal (Fig. 9).

![Fig. 9. Determination of hemolysin doses in Forssman shock. Groups of 6–8 guinea pigs were used. Mortality and lung weight were determined 10 min after injection of hemolysin at each dose. Each column for lung weight represents the mean±S.E. **P<0.01: significantly different from the control by Student's t-test.](image)

| Survival | Control | FUT-175 | Leupeptin | IDM | HC |
|----------|---------|---------|-----------|-----|----|
| >3600    |         |         |           |     |    |
| >900     |         |         |           |     |    |
| 720      |         |         |           |     |    |
| 540      |         |         |           |     |    |
| 360      |         |         |           |     |    |
| 180      |         |         |           |     |    |

| Drug     | Control | FUT-175 | Leupeptin | IDM | HC |
|----------|---------|---------|-----------|-----|----|
| Dose (mg/kg i.v.) | 0.3 | 1.0 | 3.0 | 3.0 | 10.0 | 1.0 | 3.0 |

| Rank sum test | * ** | * | ** |

![Fig. 10. Effect of intravenously administered FUT-175 on survival time in Forssman shock. Groups of 6–10 guinea pigs were used. Drugs were dosed 5 min before injection of hemolysin (0.5 ml/animal), and deaths were recorded. Each point represents the survival time of an individual guinea pig and "Survival" means that the survival time was longer than 24 hr. *P<0.05, **P<0.01: significantly different from the control by Wilcoxon's rank sum test.](image)
ii) Survival time and lung weight in Forssman shock: As described in the Methods section and taking the findings obtained in the experiments mentioned previously into consideration, doses of hemolysin employed were 0.5 ml/animal for survival time determination and 0.2 ml/animal for lung weight measurement.

Intravenous dosing of FUT-175 resulted in protection of guinea pigs from the lethal shock: 33 and 100% of the guinea pigs that received FUT-175 at doses of 1.0 and 3.0 mg/kg, respectively, could survive the 24-hr period of observation (Fig. 10), and no increase in lung weights over the intact control group were observed at doses of 1.0 and 5.0 mg/kg (Fig. 11). Moderate effects were shown by 3 and 10 mg/kg leupeptin and 1 mg/kg IDM, and no definite effect was shown by 3 mg/kg hydrocortisone (Fig. 10).

By oral administration of doses of 50, 100 and 200 mg/kg, FUT-175 was also shown to protect guinea pigs from the Forssman shock, and 2 of 10 guinea pigs dosed with 50 mg/kg survived the 20-min period of

![Fig. 11. Effect of intravenous administration of FUT-175 on lung weight change in Forssman shock. Groups of 6–10 guinea pigs were used. Drugs were dosed 5 min before injection of hemolysin (0.2 ml/animal). Lung weights were measured 10 min after injection of hemolysin. Each column represents the mean±S.E. **P<0.01: significantly different from the systemic Forssman shock group by Student’s t-test.](image)

| Survival | > 3600 | > 900 |
|----------|-------|-------|
| Drug     | Control | FUT-175 |
| Dose (mg/kg, po) | 50 | 100 | 200 |
| Rank sum test | * | * | * |

![Fig. 12. Effect of oral administration of FUT-175 on survival time in Forssman shock. Groups of 6–10 guinea pigs were used. Drugs were dosed 1 hr before injection of hemolysin (0.5 ml/animal). and deaths were recorded. Each point represents the same as described in Fig. 10. **P<0.01: significantly different from the control by Wilcoxon’s rank sum test.](image)
observation; 1, 1 and 1 of 10 dosed with 100 mg/kg survived the 20-min, 1- and 24-hr period, respectively; and 1 and 4 of 10 dosed with 200 mg/kg survived the 20-min and 24-hr period of observation, respectively (Fig. 12).

The guinea pigs that survived the 24-hr period of observation showed no typical symptoms of Forssman shock, except mild respiratory difficulties observed shortly after hemolysin injection.

iii) Effects on parameter changes: In some guinea pigs that were considered too moribund to survive 5 min after hemolysin injection, blood sampling was performed before the 5-min period elapsed. For an i.v. dose of 3.0 mg/kg, an additional group of guinea pigs were studied with a blood specimen taken 24 hr after hemolysin injection.

No remarkable change was observed in the erythrocyte count, suggesting no hemo-dilution or hemoconcentration due to the Forssman reaction, and FUT-175 administration alone at a dose of 3 mg/kg was shown not to induce any appreciable changes in the parameters studied in normal guinea pigs (Table 5, Fig. 13).

The Forssman reaction resulted in definite decreases in CH50, leucocyte counts and platelet counts and delayed blood coagulation. FUT-175 at 0.3 and 3 mg/kg was shown to normalize these changes, in a dose-dependent way, except for platelet count lowering at the determination at 5 min after, but the platelet count elevated to nearly a normal level in the group given 3 mg/kg FUT-175 at the determination performed 24 hr later (Table 5). C3 and C4 levels were also protected from decreasing by FUT-175 in a dose-dependent manner at the determination at 5 min after (Fig. 13).

2) Forssman cutaneous vasculitis

Results in Fig. 14 clearly show the dose-dependent effectiveness of FUT-175 in Forssman cutaneous vasculitis.

3. Zymosan-induced paw edema

In the control group, edema formation progressed with time, the percent edema formation being approx. 70% at the end of the 3-hr period. After i.v. dosing of FUT-175, a dose-dependent suppression of edema formation was observed, and the ED50 value was calculated as 2.4 mg/kg. FUT-175, 3 mg/kg, i.v., was shown to be more effective than IDM, 3 mg/kg, i.v. (Fig. 15).

4. Endotoxin shock

Survival rates in the control group which

---

**Fig. 13.** Effect of FUT-175 on Forssman shock-induced changes in serum C3 and C4 levels. Groups of 5–8 guinea pigs were used. Drugs were dosed intravenously 5 min before injection of hemolysin (0.5 ml/animal), and serum levels of C3 and C4 were measured 5 min after injection of hemolysin. Each column represents the mean percent level of the normal control group.
Table 5. Effects of FUT-175 on Forssman shock-induced changes of blood parameters in guinea pigs

| Animal                        | Drug   | Dose (mg/kg) | Time of determination | Parameter          |
|-------------------------------|--------|--------------|-----------------------|--------------------|
|                               |        | i.v.         |                       | Complement (CH50/ml) | Erythrocyte (10⁶/mm³) | Leucocyte (10⁹/mm³) | Platelet (10⁹/mm³) | APTT (sec) |
| Normal group                  | Control| —            | 5 min                 | 93.5±9.17          | 377±2.2             | 69±6.0             | 493±100.0         | 26.1±0.96 |
| FUT-175                       | 3.0    | 5            |                       | 116.5±6.88         | 436±14.2            | 58±5.9             | 449±33.9          | 28.3±1.25 |
| Control                       | —      | 5 min        |                       | 56.8±3.86          | 417±22.4            | 47±3.6             | 13±4.9            | 49.1±5.71 |
| Systemic Forssman shock group | FUT-175| 0.3          | 5                     | 76.2±4.91*         | 412±17.0            | 52±4.2             | 9±1.9             | 35.9±2.57 |
|                               |        | 3.0          | 5                     | 89.0±6.56**        | 361±13.0            | 56±8.3             | 21±13.2           | 33.9±2.36* |
|                               |        | 3.0          | 24 hr                 | 85.2±6.29**        | 431±13.1            | 84±6.7**           | 404±70.7**        | —          |

Groups of 5–8 guinea pigs were used. Drugs were dosed 5 min before injection of hemolysin. Blood specimens were taken 5 min after injection of hemolysin. Each value represents the mean±S.E. *P<0.05, **P<0.01: significantly different from the control by Student’s t-test.
received 40 mg/kg endotoxin, i.p., were 10 and 0% by 24 and 48 hr, respectively, after endotoxin challenge.

After i.v. dosing of FUT-175, no lowered mortalities were obtained with a dose of 3 mg/kg, but an improved survival rate was attained with a dose of 10 mg/kg: survival rates were 50% and 25% after 24 and 48 hr, respectively.

In the group receiving 10 mg/kg of gabexate mesilate, however, the mortality was nearly the same as in the control group. FUT-175 at 3 and 10 mg/kg, dosed intraperitoneally, was found to result in survival rates of 50 and 55%, respectively, after 24 hr, and 10 and 35%, respectively, after 48 hr (Table 6).

---

**Table 6. Effect of FUT-175 on endotoxin shock**

| Drug               | Dose (mg/kg) | Route | Survival rate (%) after challenge of endotoxin |
|--------------------|--------------|-------|-----------------------------------------------|
|                    |              |       | 24 hr | 48 hr |
| Control            | —            |       | 10.0  | 0     |
| FUT-175            | 3            | i.v.  | 10.0  | 0     |
|                    | 10           |       | 50.0**| 25.0* |
| Gabexate mesilate  | 10           | i.v.  | 10.0  | 0     |
|                    | 50.0**       |       | 50.0**| 10.0  |
| FUT-175            | 10           | i.p.  | 55.0**| 35.0* |

Groups of 20 mice were used. Drugs were each dosed intravenously and intraperitoneally 1 min before injection of endotoxin. Each value represents the survival rate after 24 and 48 hr following injection of endotoxin. *P<0.05, **P<0.01: significantly different from the control by the χ²-test.
5. Local Shwartzman reaction
The local Shwartzman reaction reached the maximum 3–5 hr after challenge in the control group, and FUT-175 given orally at doses of 10, 30, and 100 mg/kg clearly suppressed the reactions in a dose-dependent manner. Gabexate mesilate and IDM were also shown to be effective as depicted in Table 7.

III. Effects on trypsin
1. In vivo trypsin inhibitory effects
In the control group, blood trypsin activity level was raised progressively with time, reaching the peak at a determination of 45 min after start of infusion at the level of (102.6±15.9) × 10^{-4} μmole/min/ml. Infusion of FUT-175 at 1–25 μg/kg/min was definitely effective in trypsin inhibition, and in fact, the rise of blood trypsin activity level was observed to be inhibited dose-dependently (Fig. 16).

2. Trypsin-induced shock
In the control group, all mice were killed by i.v. injection of 0.52 mg/animal within the 2-hr period of observation.

| Drug                | Dose (mg/kg) | Index after rechallenge of LPS |
|---------------------|--------------|--------------------------------|
|                     | p.o.         |  3 hr                          |  5 hr                          |  24 hr                          |
| Control             | —            |  2.4±0.40                      |  2.4±0.56                      |  2.1±0.78                      |
| FUT-175             | 10           |  1.1±0.37*                     |  1.3±0.46                      |  1.3±0.54                      |
|                     | 30           |  0.8±0.34*                     |  0.6±0.24*                     |  0.5±0.20                      |
|                     | 100          |  0.5±0.16**                    |  0.6±0.19*                     |  0.4±0.24                      |
| Gabexate mesilate   | 100          |  1.8±0.54                      |  1.7±0.70                      |  2.0±0.57                      |
| Indomethacin        | 10           |  0.9±0.19**                    |  1.3±0.46                      |  1.0±0.41                      |

Table 7. Effect of FUT-175 on local Shwartzman reaction

Groups of 5 guinea pigs were used. Drugs were orally administered 1 hr before injection of LPS. The degree of the reaction was given one of five grades: 0, practically no change; 1, mild erythema; 2, definite erythema; 3, erythema with small necrosis and 4, necrosis. Each value represents the mean±S.E.

Fig. 16. Effect of FUT-175 on the intravenously infused trypsin activities in plasma. Groups of 5–6 rabbits were used. Drugs and trypsin were simultaneously drip-infused for 60 min through each right and left marginal ear vein, and BocPheSerArgMCA-hydrolytic activities in plasma were measured. Each point represents the mean±S.E. *P<0.05, **P<0.01: significantly different from the control by Student's t-test.
FUT-175 was effective in lowering mortalities in a dose-dependent way, and thus, although a dose of 1.0 mg/kg, i.v. was ineffective, doses of 3.0 and 10 mg/kg, i.v., resulted in survival rates of 41.6 and 83.3%, respectively, ED50 in the trypsin-induced shock being 4.17 mg/kg, i.v. (Table 8).

On the other hand, aprotinin at 10×10^5 KIU/kg, i.v. and gabexate mesilate at 10 mg/kg, i.v. were found to have practically no effect, and thus, the survival rates observed were 20 and 0%, respectively.

IV. Effects on thrombin
1. In vivo thrombin inhibitory effects
As illustrated in Fig. 17, FUT-175 was clearly effective, particularly at doses of 10 and 100 μg/kg/min for 1 hr, in preventing the lowering of plasma fibrinogen levels caused by thrombin infusion.

2. Effects on thrombin-thrombosis
After intravenous injection of 300 U/kg of thrombin, all deaths observed occurred within 1 hr following thrombin injection. FUT-175 effectively prevented deaths due to thrombin-thrombosis, and in fact, 6 mice out of 10 given FUT-175 at 3 and 10 mg/kg, respectively, could survive the 24-hr period of observation.

Gabaxate mesilate at 10 mg/kg, however, was ineffective, and heparin at 100 U/kg protected the mice completely from death (Table 9).

V. Effects on the kinin formation system
1. Kinin formation in plasma
   i) In vitro experiments: Results are presented in Table 10. As the table shows, FUT-175 inhibited the kinin formation in plasma with the following IC50s: 1.1×10^{-7} M (rat) and 5.5×10^{-7} M (dog) for glass powder and 3.8×10^{-6} M (rat) and 3.7×10^{-6} M (dog).
Pharmacological Studies of FUT-175

Pharmacological Studies of FUT-175

Kinin formation in plasma in the absence of FUT-175 were as follows (ng BK eq/ml): 63±0.4 (rat) and 39±3.6 (dog) for glass powder and 56±5.8 (rat) and 56±2.2 (dog) for trypsin.

In the system of rat plasma-rat submandibular kallikrein preparation of 1:160

Table 9. Effect of FUT-175 on thrombin-induced thrombosis

| Drug            | Dose (mg/kg) | Survival rate (%) |
|-----------------|--------------|-------------------|
|                 | i.v.         |                   |
| Control         | —            | 10.0              |
| FUT-175         | 0.3          | 10.0              |
|                 | 1.0          | 40.0              |
|                 | 3.0          | 60.0**            |
|                 | 10           | 60.0**            |
| Gabexate mesilate | 10           | 20.0              |
| Heparin         | 100 U        | 100 **            |

Groups of 10 mice were used. Drugs were dosed 1 min before injection of thrombin. Each value represents the mean survival rate after 24 hr following injection of thrombin. **P<0.01: significantly different from the control by the χ²-test.

Table 10. In vitro effect of FUT-175 on the kinin formation

| Concentration of FUT-175 (M) | Glass powder (100 mg/ml) | Trypsin (1 mg/ml) | Glandular kallikrein |
|------------------------------|--------------------------|-------------------|----------------------|
|                              | Rat          | Dog          | Rat           | Dog         | Rat          |
| 1×10⁻⁸                       | 14.2         | —            | —             | 1.1         | —            |
| 1×10⁻⁷                       | 45.5         | 1.3          | 14.3          | —           | —            |
| 3×10⁻⁷                       | 86.8         | 9.4          | 16.7          | —           | —            |
| 1×10⁻⁶                       | 100.0        | 76.6         | 24.2          | 0.2         | 20.8         |
| 3×10⁻⁶                       | —            | —            | —             | 30.2        | —            |
| 1×10⁻⁵                       | —            | 99.0         | 96.3          | 94.8        | 65.1         |
| 1×10⁻⁴                       | —            | —            | —             | 99.6        | 93.5         |

Plasma samples were from dogs or rats, and the kinin-generating substances were glass powder, trypsin and glandular kallikrein. Assay of the kinin formed was achieved by determining the uterus contraction-inducing potency in comparison with bradykinin. Each value represents percent inhibition of the control.

Table 11. Ex vivo effect of FUT-175 on the kinin formation in rats

| Drug  | Dose (mg/kg) | Route | No. of animal | Kinin formation (ng BK eq/ml plasma) |
|-------|--------------|-------|---------------|--------------------------------------|
| Control | —           | i.v.   | 5             | 50.0±5.9                             |
| FUT-175 | 0.1         | 6     | 32.9±3.0*     |
|        | 0.3         | 6     | 21.5±3.7**    |
|        | 1.0         | 6     | 11.6±2.9**    |

Groups of 5–6 rats were used. The plasma specimens were obtained 5 min after intravenous dosing of FUT-175. Kinin formation in the plasma specimen was performed using glass powder as the kinin-generating substance. Each value represents the mean±S.E. *P<0.05. **P<0.01: significantly different from the control by Student’s t-test.

for trypsin.

Kinin formation in plasma in the absence of FUT-175 were as follows (ng BK eq/ml): 63±0.4 (rat) and 39±3.6 (dog) for glass powder and 56±5.8 (rat) and 56±2.2 (dog) for trypsin.
dilution, kinin formation was found to be 68±11.2 ng BK eq/ml and FUT-175 was also effective in inhibiting the kinin formation with an IC50 of 5.5×10^{-6} M.

ii) Ex vivo experiments: As shown in Table 11, kinin formation by glass powder in the control group was 50±5.9 ng BK eq/ml, and FUT-175 inhibited this in a dose-dependent way.

2. Kinin formation in inflammatory process

In the control group, kinin activity of the pre-perfusates was 0.95±0.23 ng BK eq/ml, and those in the test-perfusates were 1.34±0.15, 1.22±0.16 and 1.01±0.23 ng BK eq/ml for the 1st, 2nd and 3rd 15-min period of collection, respectively, the peak level being found in the test-perfusate of the 1st 15-min period.

FUT-175 was effective in inhibiting dose-dependently the rise of kinin activity in the perfusates caused by heating at 46°C, and at a dose of 3 mg/kg, kinin activity levels in the perfusates during 45 min were, as a whole, lower than in the pre-perfusates (Fig. 18).

Discussion

As the results of the experiments to study the effects of FUT-175 on various proteases, it has been found that FUT-175 has a specific and intense inhibitory activity on C1r, C1s, trypsin, thrombin, plasmin and kallikrein, all classified as trypsin-like serine-proteases which are known to have a substrate specificity for arginyl and lysyl residue-containing substrates. Thus, FUT-175 has been shown to exhibit a weak or practically no inhibition against other proteases studied, namely, chymotrypsin, belonging to the class of serine-proteases which have a substrate specificity for phenylalanyl, tyrosyl and tryptophan residue-containing substrates; elastase, belonging to the class of serine-proteases which have a substrate specificity for alanyl residue-containing substrates; papain, a thiol-protease; collagenase, a metalloprotease; pepsin and cathepsin D, both acid proteases.

The findings that FUT-175 has potent anticomplement activity with an IC50 of 10^{-7}–10^{-8} M against C1r and C1s and is practically devoid of cathepsin D-inhibitory activity are of particular interest.

It has also been found that phospholipase A2 is moderately inhibited with an IC50 of 10^{-5} M order and that lipase is practically not inhibited by FUT-175.

The kinetic study on FUT-175 inhibition against proteases using Lineweaver-Burk plots has shown that the inhibition against C1r, thrombin, plasmin and kallikrein is a competitive one and that against C1s and trypsin, it is a non-competitive one; the $K_i$ values are in the range of 10^{-7}–10^{-8} M, indicating high affinity of FUT-175 to these proteases. Dialysis studies conducted on each trypsin- and thrombin–FUT-175 complex have revealed that the esterolytic activity of thrombin, being inhibited by FUT-175 in a competitive manner, is readily recovered to 100% of the initial activity after an overnight
Pharmacological Studies of FUT-175

Dialysis at 4°C, and the esterolytic activity of trypsin, being inhibited by FUT-175 in a non-competitive manner, on the other hand, is recovered by approx. 20% after an overnight dialysis at 4°C and 100% by an additional incubation for 5 hr at 37°C.

Markwardt et al. (22) have described that corresponding to the mechanism of action, there are three different types of inhibitors, namely, (1) competitive inhibitors which form reversible complexes with the enzyme, the effectiveness of this type of inhibitors being characterized by its affinity to the enzyme; (2) temporary inhibitors which react with the enzyme like substrate, forming rapidly an acyl-enzyme followed by a very slow deacylation; and (3) irreversible inhibitors which are covalently linked to the active site of the enzyme, but the acyl-enzyme formed in this way is stable and no deacylation takes place.

NPGB (p-nitrophenyl p-guanidinobenzoate) has been shown to be a potent temporary inhibitor of serum kallikrein by Markwardt et al. (op. cit.) and shown to be a preferable active site titrant of trypsin-like enzymes by Chase, Jr. and Shaw (23). Diisopropyl fluorophosphate, DFP, is known to inhibit serine-proteases in a non-competitive manner and not to be removed from the enzyme-DFP complex by dialysis.

Taking these into consideration, the difference in the rate of deacylation from the acyl-enzymes formed by an action of FUT-175 on proteases is speculated to result in apparently different types of kinetics, namely, the competitive inhibition and the non-competitive inhibition.

It is well known that serum contains α2-macroglobulin with which trypsin forms a complex. This trypsin-α2MG complex retains enzymatic activity and has been shown to play an important role in pancreatitis (24). The susceptibility of the trypsin in the complex to soybean trypsin inhibitor is practically lost, and susceptibility to aprotinin is decreased to a level which is 1/16 that of free trypsin. FUT-175 has been, however, found to be effective to the same extent against both free trypsin and the trypsin in the α2MG complex.

FUT-175 has been found to inhibit strongly the in vitro complement-mediated hemolysis with an IC50 of 3.0×10⁻⁸ M for the classical pathway activation and of 3.0×10⁻⁷ M for the alternative pathway activation. Compounds having anti-complement activity have been reported, for example, benzamidines by Hauptmann and Markwardt (25), meta-substituted benzamidines and benzoyl halide-substituted pyridines by Baker and Cory (26), maleo- and fumaro-pimaric acids by Glovsky et al. (op. cit.), K-76-COOH by Hong et al. (27) and complestatin by Kaneko et al. (28), both being microbial products, and peptidyl inhibitors recently reported by Lukas et al. (29), Lesavre et al. (30) and others (31, 32). The results obtained in the present study show that FUT-175 is ranked as one of the most potent anticomplement agents, even considering differences in the conditions of determination.

FUT-175 inhibition of the complement-mediated hemolysis was attributable its complement inhibitory action because the complement concentration-hemolysis curve was shifted to the right in parallel in the presence of FUT-175. A study using undiluted serum has shown us that FUT-175, a compound having an ester linkage, is fairly stable in the serum in that a preincubation of FUT-175 in undiluted guinea pig serum at 37°C for 10 min does not result in remarkable loss of hemolysis-inhibitory effectiveness, shortening of T50 being only approx. 15% and increase of S being approx. 25%.

In the experiments to study the sites of FUT-175 inhibition of complement-mediated hemolysis, it was demonstrated that hemolysis was inhibited in the systems in which FUT-175 was added to the incubation mixture of EAC4 and C1 and to that of EAC14 and C2. In activation of the complement system via the classical pathway, C1γ, formed from C1s by the action of C1f which is generated by spontaneous activation of C1r as the result of binding of the immune-complex to C1q, cleaves C4 into C4a and C4b and cleaves C2 into C2a and C2b, and C4b2a thus formed cleaves C3 to form C3a and C3b (33). FUT-175 inhibition of the complement-mediated hemolysis by the classical pathway activation is, consequently, considered to be produced by inhibition of
C1r and C1s as expected from the results that FUT-175 inhibited the esterolytic activity of C1r and C1s on synthetic substrates.

FUT-175 inhibition of alternative pathway-activated hemolysis has been shown by Ikari et al. (34) to be attributed to its inhibition against B, D, Bb and C3 convertase (C3bBb).

The in vivo inhibitory effectiveness of FUT-175 on complement-mediated hemolysis was also studied. In the in vivo model of complement-mediated hemolysis via the classical pathway, which was undoubtedly shown by the finding that no hemolysis occurred after i.v. injection of unsensitized SRBC to normal guinea pigs, FUT-175 was found to be effective in hemolysis inhibition, in a dose-dependent manner, by both i.v. and p.o. routes, though being much less effective by the latter route. In the in vivo complement-mediated hemolysis via the alternative pathway, which was also evidenced by the finding that the inactivated dog serum at 56°C for 30 min induced no hemolysis, FUT-175 was effective by both the i.v. route and p.o. route, the latter being also much less active.

Effectiveness of FUT-175 has been evaluated in various animal model reactions in which the complement system is known to be involved as one of the pathogenetic or etiological factors, and those studied were systemic Forssman shock, Forssman cutaneous vasculitis, zymosan-induced paw edema, endotoxin shock and local Shwartzman reaction.

The Forssman reaction is considered to be an appropriate animal model reaction for studying the in vivo effectiveness of any compounds having anticomplement action, for the early components of the complement system play an important etiological role as shown by Frank et al. (35) who demonstrated that this reaction could not be provoked in C4-deficient guinea pigs, and reconstitution of the classical pathway by the administration of normal guinea pig serum as a source of C4 made C4-deficient animals susceptible to the Forssman reaction.

Zymosan is known to activate the complement cascade via the alternative pathway and the complement system has been speculated to play important roles in zymosan-induced acute paw edema by Gemmell et al. (36) and in zymosan-induced exudative inflammation in an air pouch by Konno and Tsurufuji (37). Endotoxin is also known to activate the complement system via the alternative pathway and to cause deaths of animals by endotoxin shock with high enough doses (38, 39).

Contribution of the complement system in the Shwartzman reaction is well known, and it has widely been described that endotoxin activates the classical pathway by means of an endotoxin-endotoxin antibody complex, the antibody to endotoxin being present even in normal serum in a small amount, and activates directly the alternative pathway to induce the Shwartzman reaction.

Before the study on FUT-175 effectiveness in systemic Forssman shock, determinations were made of the optimal doses of hemolysin for provoking lethal shock and increasing of lung weight due to edema formation and hemorrhage, and doses of 0.5 ml/animal for the former and 0.2 ml/animal for the latter were chosen.

Effectiveness of i.v. dosing of FUT-175 was particularly remarkable, and in fact, a dose as low as 3 mg/kg could protect all guinea pigs tested from the lethal shock, and they could survive the 24-hr period of observation. Fumaropimaric acid has been so far reported by Glovsky et al. (op. cit.) to be effective in protecting guinea pigs from lethal shock, but the effective dose by i.v. injection was as high as 200 mg/kg and near the toxic level. FUT-175 was also demonstrated to suppress completely the increase in lung weights at doses higher than 1 mg/kg given intravenously.

FUT-175 at 3 mg/kg, i.v., causing no appreciable changes in the parameters studied in normal guinea pigs, could evidently bring about a normalization of the parameter changes in the Forssman reaction, and thus, lowering of both C3 and C4 levels was much lessened in a dose-dependent way. In Forssman cutaneous vasculitis, FUT-175 was also effective.

FUT-175 has been shown to be markedly and dose-dependently effective by intravenous dosing in zymosan-induced paw edema in rats and endotoxin shock in mice.
and by oral administration in the local Shwartzman reaction.

Thus, the present study has proved that FUT-175, having potent in vitro inhibitory activity on C1r, C1s, B, D and C3bBb, has a noticeable in vivo effectiveness in various animal model reactions in which the complement system contributes to large extent as a pathogenetic factor.

Considering the strong inhibition of esterolytic and caseinolytic activity of trypsin by FUT-175 in the in vitro experiments, animal experiments were carried out in an attempt to evaluate the in vivo trypsin inhibitory effectiveness of FUT-175. It was shown to be inhibitory against trypsin in the in vivo experiments: the higher the drip infusion dose of FUT-175 was, the lower the level of BocPheSerArgMCA hydrolytic activity in the circulation, presumably corresponding to the exogenously administered trypsin, was, and the mice were protected from the trypsin-induced lethal shock, in a dose-dependent manner, by i.v. injection of FUT-175.

FUT-175, having potent in vitro antithrombin activity, has been also shown to be effective in vivo in suppressing thrombin action, and it prevented the thrombin-induced fibrinogen consumption in rabbits and the thrombin-induced deaths, presumably due to thrombotic embolism, in mice.

FUT-175 was also found to be effective in in vitro, ex vivo and in vivo experiments relevant to the kinin formation system, corresponding to the finding that FUT-175 inhibited the in vitro TAME hydrolytic activity of pancreatic kallikrein with an IC50 of 3.9×10⁻⁶ M.

In the present studies, rat submandibular gland kallikrein preparation was obtained by our own procedures, considering those published by Uchida et al. (40) and Brandtzaeg et al. (41), and the preparation was confirmed to practically contain no trypsin activity and kininase activity.

The results of the series of investigations reported in the present paper show that FUT-175 has a potent and selective inhibitory activity against C1r, C1s, trypsin, thrombin, kallikrein and plasmin in the in vitro experiments as well as in the ex vivo and in vivo experiments. Considering these basic pharmacological profiles, it is considered worthwhile to do further studies on FUT-175, for example, on effectiveness in the SLE model in NZB/W mice, the acute pancreatitis model, the nephrotoxin nephritis in rats, the endotoxin-induced DIC model, and on effects in the blood coagulation-fibrinolysis system and in inflammation models of various types. FUT-175 also deserves to be clinically studied in the diseases in which these proteases are know to be involved as pathogenetic factors.

References
1 Fujii, S. and Hitomi, Y.: New synthetic inhibitors of C1r, C1 esterase, thrombin, plasmin, kallikrein and trypsin. Biochim. Biophys. Acta 661, 342–345 (1981)
2 Hitomi, Y. and Fujii, S.: Inhibition of various immunological reactions in vivo by a new synthetic complement inhibitor. Int. Arch. Allergy Appl. Immunol. 69, 262–267 (1982)
3 Okamura, K. and Fujii, S.: Isolation and characterization of different forms of C1r, a subcomponent of the first component of human complement. Biochim. Biophys. Acta 534, 258–266 (1978)
4 Okamura, K., Muramatsu, M. and Fujii, S.: Purification of C1s, a subunit of the first component of complement from human plasma. Biochim. Biophys. Acta 295, 252–257 (1973)
5 Roberts, P.S.: Measurement of the plasmin action on synthetic substrates. J. Biol. Chem. 232, 285–291 (1958)
6 Muramatsu, M., Onishi, T., Makino, S., Fujii, S. and Yamamura, Y.: Inhibition of caseinolytic activity of plasmin by various synthetic inhibitors. J. Biochem. 57, 402–408 (1965)
7 Cunningham, M. and Tang, J.: Purification and properties of Cathepsin D from porcine spleen. J. Biol. Chem. 251, 4528–4563 (1976)
8 Morita, T., Kato, H., Iwanaga, S., Takada, T., Kimura, T. and Sakakibara, S.: New fluorogenic substrates for α-thrombin, factor Xa, kallikrein and urokinase. J. Biochem. 82, 1495–1498 (1977)
9 Yasigawa, S., Morita, F., Nagai, Y., Noda, H. and Ogura, Y.: Kinetic studies on the action of collagenase. J. Biochem. 58, 407–416 (1965)
10 Kurooka, S. and Kitamura, T.: Properties of serum lipase in patients with various pancreatic diseases. J. Biochem. 84, 1459–1466 (1978)
11 De Haas, G.H., Postema, N.M., Nieuwenhuizen, W. and Doenen, L.L.M.: Effect of phospholipase
A and pre-Phospholipase A on block phospholipid membranes. Biochim. Biophys. Acta 159, 103–109 (1968)

12 Lineweaver, H. and Burk, D.: Determination of enzyme dissociation constants. J. Am. Chem. Soc. 56, 658–666 (1934)

13 Dixon, M.: The determination of enzyme inhibitor constants. Biochem. J. 55, 170–171 (1953)

14 Nishioka, K., Tachibana, T., Okada, H., Mukojima, T., Torisu, M., Arata, M., Ohmi, K. and Kamiyama, Y.: Methods in complement study. Protein, Nucleic Acid and Enzyme 11, 1605–1619 (1966) (in Japanese)

15 Mayer, M.M.: Complement and complement fixation. In Experimental Immunochemistry, Edited by Kabat, E.A. and Mayer, M.M., ed. 2, p. 133–157, Charles C. Thomas Publisher, Springfield (1961)

16 Plescia, O.J., Amiriaian, K. and Heiderberger, M.: A kinetic method for the titrations of complement. Arch. Biochem. Biophys. 62, 346–354 (1956)

17 Platts-Mill, T.A.E. and Ishizaka, K.: Activation of the alternate pathway of human complement by rabbit cells. J. Immunol. 113, 348–358 (1974)

18 Glovsky, M.M., Ward, P.A., Becker, E.L. and Halbrook, N.J.: Role of fumaropimaric acid in guinea-pig complement dependent and non-complement dependent biologic reactions. J. Immunol. 102, 1–14 (1969)

19 Kitamura, H.: Titration method for hemolytic activities and protein concentration of complement components Clin. Immunol. 13, 161–175 (1981) (Abs. in English)

20 Litchfield, J.T. and Wilcoxon, F.: A simplified method of evaluating dose-effect experiment. J. Pharmacol. Exp. Ther. 96, 99–113 (1949)

21 Garcia, J., Hamamura, L. and Rochaesilva, M.: Effect of anti-protease and hexadimethrine bromide on the release of a bradikinin-like substance during heating (46°C) of rat paws. Br. J. Pharmacol. 40, 294–309 (1970)

22 Markwardt, F., Drawert, J. and Walsmann, P.: Synthetic low molecular weight inhibitors of serum kalikrein. Biochem. Pharmacol. 23, 2247–2256 (1974)

23 Chase, T. Jr. and Shaw, E.: Titration of trypsin, plasmin and thrombin with p-nitrophenyl p-guanidinobenzoate HCl. In Methods Enzymol. Edited by Perlmann, G.E. and Loraud, L., Vol. XIX, p. 20–27, Academic Press, New York and London (1970)

24 Harverback, B.J., Dyce, B., Bundy, H.F., Wirthafer, S.K. and Edmondson, H.A.: Protein binding of pancreatic proteolytic enzymes. J. Clin. Invest. 41, 972–980 (1970)

25 Hauptmann, J. and Markwardt, F.: Inhibition of the hemolytic complement activity by derivatives of benzamidine. Biochem. Pharmacol. 26, 325–329 (1977)

26 Baker, B.R. and Cory, M.: Irreversible enzyme inhibitors. CLXIV. Proteolytic enzymes. XIV. Inhibition of guinea pig complement by meta-substituted benzamidines. J. Med. Chem. 12, 1049–1052 (1969)

27 Hong, K., Kinoshita, T., Miyazaki, W., Izawa, T. and Inoue, K.: An anti-complementary agent, K-76 monocarboxylic acid: Its site and mechanism of inhibition of complement activation cascade. J. Immunol. 122, 2418–2423 (1979)

28 Kaneko, I., Fearon, D.T. and Austen, K.F.: Inhibition of the alternative pathway of human complement in vitro by a natural microbial product, Complementin. J. Immunol. 122, 1194–1198 (1980)

29 Lukas, T.J., Munoz, H. and Erickson, B.W.: Inhibition of C1-mediated immune hemolysis by monomeric and dimeric peptides from the second constant domain of human immuno-globulin G. J. Immunol. 127, 2555–2560 (1981)

30 Lesavre, P., Gaillard, M.H. and Halwachs-Mecarelli, L.: Inhibition of alternative pathway factor D by factor B related synthetic hexapeptides. Eur. J. Immunol. 12, 252–254 (1982)

31 Otterness, I.G., Torchia, A.J. and Doshan, H.D.: Complement inhibition by amidines and guanidines—in vivo and in vitro results. Biochem. Pharmacol. 27, 1873–1878 (1978)

32 Burge, J.J., Fearon, D.T. and Austen, K.F.: Inhibition of the alternative pathway of complement by gold sodium thiomalate in vitro. J. Immunol. 120, 1625–1630 (1978)

33 Nagasawa, S.: Protein chemistry of the complement system. Protein, Nucleic Acid and Enzyme 28, 19–44 (1983) (in Japanese)

34 Ikari, N., Sakai, Y., Hitomi, Y. and Fujii, S.: New synthetic inhibitor to the alternative complement pathway. Immunology 49, 685–691 (1983)

35 Frank, M.M., Joseph, M., Gaither, T. and Ellman, L.: In vitro studies of complement function in sera of C4-deficient guinea pigs. J. Exp. Med. 134, 176–187 (1971)

36 Gemmell, D.K., Cottney, J. and Lewis, A.J.: Comparative effects of drugs on four paw edema models in the rat. Agents Actions 9, 107–116 (1979)

37 Konno, S. and Tsurufuji, S.: Induction of zymosan-air-pouch inflammation in rats and its characterization with reference to the effects of anti-complementary and anti-inflammatory agents. Br. J Pharmacol. 80, 269–277 (1983)
38 Gewurz, H., Shin, S.H. and Mergenhagen, S.E.: Interaction of the complement system with endotoxic lipopolysaccharide: consumption of each of the six terminal complement components. J. Exp. Med. 128, 1049–1057 (1968)

39 Ulevitch, R.J., Cochrane, C.G., Henson, P.M., Morrison, D.C. and Doe, W.F.: Mediation systems in bacterial lipopolysaccharide-induced hypotension and disseminated intravascular coagulation I. The role of complement. J. Exp. Med. 142, 1570–1590 (1975)

40 Uchida, K., Niinobe, M., Kato, H. and Fujii, S.: Purification and properties of rat stomach kallikrein. Biochim. Biophys. Acta 614, 501–510 (1980)

41 Brandtzaeg, P., Gentvic, K.M., Nustad, K. and Pierce, J.V.: Rat submandibular gland kallikreins: Purification and cellular localization. Br. J. Pharmacol. 56, 155–167 (1976)