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Biochemical and pharmacological characterization of FK706, a novel elastase inhibitor

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Abstract

FK706, sodium 2-[4-[[(*S*)-1-[[(*S*)-2-[[(*RS*)-3, 3, 3-trifluoro-1-isopropyl-2-oxopropyl]aminocarbonyl]pyrrolidin-1-yl]carbonyl]-2-methylpropyl] aminocarbonyl] benzoylamino] acetate, $C_{26}H_{32}F_3N_4NaO_7$, is a synthetic water-soluble inhibitor of human neutrophil elastase. This compound demonstrated a competitive and slow-binding inhibition of human neutrophil elastase with a K_i of 4.2 nM. In studies using synthetic substrates, FK706 inhibited human neutrophil elastase activity and porcine pancreatic elastase activity with respective IC_{50} values of 83 and 100 nM. FK706, however, inhibited more weakly, (IC_{50} values > 340 μM) other serine proteinases such as human pancreatic α-chymotrypsin, human pancreatic trypsin and human leukocyte cathepsin G. FK706 also effectively inhibited the hydrolysis of bovine neck ligament elastin (2 mg/ml final concentration) by human neutrophil elastase (4 μg/ml final concentration) with an IC_{50} value of 230 nM. FK706 protected animals against human neutrophil elastase (50 μg/animal)-induced lung hemorrhage with ED_{50} values of 2.4 μg/animal by intratracheal administration and 36.5 mg/kg by intravenous administration, respectively. Subcutaneous administration of FK706 significantly suppressed human neutrophil elastase (20 μg/paw)-induced paw edema in mice in a dose-dependent manner (47% inhibition at a dose of 100 mg/kg). These results suggest that FK706 would be a useful tool for investigating the role of human neutrophil elastase in inflammatory disorders associated with an excess of elastase, such as pulmonary emphysema, adult respiratory distress syndrome, septic shock, cystic fibrosis, chronic bronchitis and rheumatoid arthritis. © 1997 Elsevier Science B.V.

Keywords: FK706; Elastase inhibitor; Neutrophil elastase; Pulmonary emphysema; Hemorrhage; Edema

1. Introduction

Elastases are part of a family of serine proteinases that hydrolytically degrade connective tissue components such as elastin, proteoglycan, fibronectin and collagen types I, II, III and IV (Havemann and Gramse, 1984). The protein, elastin, is an essential, highly flexible and highly hydrophobic component of lung connective tissue, arteries, skin and ligaments. Human neutrophil elastase, which is stored in the azurophilic granules of polymorphonuclear leukocytes and is released by inflammatory stimuli, is considered to be the primary source of tissue damage associated with such inflammatory diseases as pulmonary emphysema (Janoff, 1985; Groutas, 1987), adult respiratory distress syndrome (Lee et al., 1981; McGuire et al., 1982), septic shock (Uchida et al., 1995), cystic fibrosis (O'Connor et al., 1993; Hansen et al., 1995), chronic bronchitis (Llewellyn-Jones et al., 1996), rheumatoid arthritis (Mohr and Wessinghage, 1983) and other inflammatory states (Adeyemi et al., 1985; Fric et al., 1985).

Extracellular human neutrophil elastase released from the leukocytes is normally inhibited by endogenous inhibitors such as α1-proteinase inhibitor, so that its physiological action is restricted. Recently, it has been postulated that pulmonary emphysema occurs as a result of a local elastase–anti-elastase imbalance caused by oxidative inactivation or a genetic deficiency of α1-proteinase inhibitor (Carp et al., 1982; Cox and Levison, 1988; Gadek and Pacht, 1990). Indeed, individuals deficient in α1-proteinase inhibitor (Pi_{zz} phenotype) are known to be particularly susceptible to early development of emphysema (Larsson, 1978). Evidence has also been reported that it may be involved in the pathogenesis of increased and

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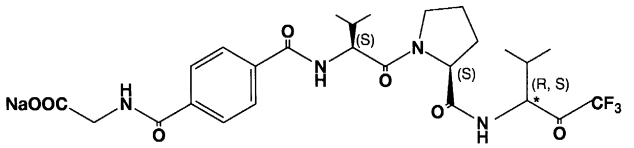

Fig. 1. Chemical structure of FK706, sodium 2-[4-[[(*S*)-1-[[(*S*)-2-[[(*RS*)-3, 3, 3-trifluoro-1-isopropyl-2-oxopropyl]aminocarbonyl]pyrrolidin-1-yl]carbonyl]-2-methylpropyl]aminocarbonyl]benzoylamino] acetate, $C_{26}H_{32}F_3N_4NaO_7$.

abnormal airway secretions commonly associated with airway inflammatory diseases (Sommerhoff et al., 1991). Thus, bronchoalveolar lavage fluid from patients with chronic bronchitis and cystic fibrosis had increased neutrophil elastase activity. More recently, elastase inhibitors were reported to prevent antigen-induced bronchoconstriction in an animal model of asthma (Fujimoto et al., 1995). Furthermore, excessive elastase has been proposed to contribute not only to these chronic inflammatory diseases but also to acute inflammatory diseases such as adult respiratory distress syndrome and septic shock. These findings have stimulated interest in the search for agents that have elastase inhibitory activity and many low molecular mass synthetic inhibitors of human neutrophil elastase have been described and reviewed (Fletcher et al., 1990; Kawabata et al., 1991; Williams et al., 1991a,b).

We recently synthesized a novel, water-soluble elastase inhibitor, FK706, sodium 2-[4-[[(*S*)-1-[[(*S*)-2-[[(*RS*)-3, 3, 3-trifluoro-1-isopropyl-2-oxopropyl] aminocarbonyl] pyrrolidin-1-yl] carbonyl]-2-methylpropyl] aminocarbonyl] benzoylamino] acetate, $C_{26}H_{32}F_3N_4NaO_7$. FK706 consists of a trifluoromethyl ketone moiety with a molecular mass of 592.55 Da (Fig. 1). In the present study, we have examined enzymatic characteristics of FK706, a potent, slow-binding and competitive inhibitor of human neutrophil elastase by using both synthetic and natural substrates and have examined the effects of FK706 on human neutrophil elastase-induced lung hemorrhage and paw edema in experimental animals.

2. Materials and methods

2.1. Animals and reagents

Human sputum elastase (EC 3.4.21.37) (875 units/mg protein using succinyl–Ala–Ala–*p*-nitroanilide as a substrate) and bovine neck ligament elastin congo-red were purchased from Elastin Products (Pacific, MO, USA). Human sputum elastase was used as human neutrophil elastase without further purification (Skiles et al., 1984; Fletcher et al., 1990; Green et al., 1991). Mouse elastase was prepared as a supernatant after hypo-osmotic treatment of neutrophils from the peritoneal cavity in the ICR (Institute of Cancer Research, USA) strain mice that received 0.5% glycogen i.p. Porcine pancreatic elastase (EC 3.4.21.36) (type IV, 69 units/mg protein using elastin as a substrate), human pancreatic α-chymotrypsin (EC 3.4.21.1) (61.6 BTEE units/mg protein using *n*-benzoyl–Tyr–ethyl-ester (BTEE) as a substrate), human pancreatic trypsin (EC 3.4.21.4) (12 600 BAEE units/mg protein using *n*-benzoyl–Arg–ethyl-ester (BAEE) as a substrate), human leukocyte cathepsin G (EC 3.4.21.20) (60 units/mg protein using *n*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide as a substrate), methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide, *n*-succinyl–Ala–Ala–Ala–*p*-nitroanilide, methoxysuccinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide, *n*-benzoyl–Arg–*p*-nitroanilide and α1-proteinase inhibitor (2.8 mg inhibit 1.0 mg of trypsin with activity of 10 000 BAEE units/mg protein, 3.5 mg inhibit 1.0 mg of α-chymotrypsin with activity of 40–50 BAEE units/mg protein) were purchased from Sigma (St. Louis, MO, USA). FK706 and ONO-5046 (*N*-[2-[4-(2,2-dimethylpropionyloxy)phenylsulfonylamino]benzoyl]aminoacetic acid) were synthesized in our laboratory. Other chemicals were of reagent grade and 96-well microtiter plates (MS3496FE) were purchased from Sumitomo Bakelite (Tokyo, Japan). Female golden hamsters (*Mesocricetus auratus*) aged five weeks, weighing approximately 80 g were obtained from Charles River (Wilmington, MA, USA). Male C57BL mice aged 6 weeks were obtained from Clea Japan (Tokyo, Japan).

2.2. Determination of K_i value for human neutrophil elastase

The K_i value was determined according to a method previously described, with some modifications (Cha, 1975; Shapiro and Riordan, 1984; Stein et al., 1987). Briefly, reaction progress was measured spectrophotometrically by monitoring the release of *p*-nitroaniline at 410 nm with a Hitachi U-3200 spectrophotometer during the hydrolysis of methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide at 37°C. 3 ml of reaction mixture contained 2370 μl of 0.1 M HEPES, 0.5 M NaCl pH 7.5, 300 μl of substrate dissolved in the same buffer containing 30% DMSO (dimethyl sulfoxide) (final substrate concentration; 600 μM, final DMSO concentration 3%) and 300 μl of inhibitor (FK706) dissolved in the same buffer. The reaction was started by adding 30 μl of 10 μg/ml enzyme solution (final enzyme concentration; 3.3 nM). Absorbance was continuously monitored. Initial and steady state velocities were calculated by a fitting the data to a linear dependence on time by linear-squares analysis. Duplicate measurements were made for each inhibitor concentration.

2.3. Determination of IC_{50} values for proteinases by using synthetic substrates

IC_{50} values for various proteinases were determined using a minor modification of the method described earlier (Bonney et al., 1989). Briefly, total 200 μl of incubation mixture containing 50 μl of inhibitor, 100 μl of 1 mM substrate dissolved in 1% DMSO (added last, final sub-

strate concentration; 0.5 mM, final DMSO concentration; 0.1%) and 50 μl of enzyme in 0.1 M HEPES, 0.5 M NaCl, pH 7.5 was incubated on a 96-well microtiter plate at 37°C for 3 h. After incubation, the release of *p*-nitroaniline was assayed by the absorbance at 405 nm on a BIO-RAD Model 3550 Microplate Reader. Inhibitory activities of each inhibitor against various proteinases were evaluated from the increases in optical density at 405 nm. Human neutrophil elastase at a final concentration of 32 ng/ml was assayed using methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide as a substrate. Porcine pancreatic elastase at a final concentration of 40 ng/ml was assayed using *n*-succinyl–Ala–Ala–Ala–*p*-nitroanilide (Fujimoto et al., 1980) as a substrate, human α-chymotrypsin at a final concentration of 10 ng/ml and human neutrophil cathepsin G at a final concentration of 320 ng/ml were assayed using methoxysuccinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide (Wilcox, 1970; Barrett, 1981) as a substrate and trypsin at a final concentration of 200 ng/ml was assayed using *n*-benzoyl–Arg–*p*-nitroanilide (Lee et al., 1987) as a substrate. Mouse neutrophil elastase was also assayed using methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide as a substrate. Since mouse elastase was a crude preparation, we measured the protein concentration of mouse elastase so that the optical density at 405 nm could be obtained in the same way as that of human neutrophil elastase under our assay conditions. Triplicate measurements were conducted for each inhibitor concentration for each enzyme.

2.4. Determination of IC_{50} values for human neutrophil elastase by using insoluble elastin

The ability of FK706 and α1-proteinase inhibitor to inhibit the hydrolysis of insoluble elastin by human neutrophil elastase was determined spectrophotometrically using elastin congo-red as a substrate according to the method of previous report with some modifications (Naughton and Sanger, 1961). Briefly, elastin congo-red (final concentration; 2 mg/ml) and human neutrophil elastase (final concentration; 4 μg/ml) were incubated with various concentrations of FK706 or α1-proteinase inhibitor in 1.5 ml of 0.1 M Tris–HCl buffer pH 8.0 containing 0.2 M of NaCl at 30°C for 1 h. After incubation, the reaction was stopped by adding 1.5 ml of 0.1 M acetic acid and the mixture was centrifuged at 3000 rpm for 10 min at room temperature. After centrifugation, the absorbance at 495 nm of the supernatant was measured with a Hitachi U-3200 spectrophotometer. Triplicate measurements were made for each inhibitor concentration.

2.5. Human neutrophil elastase-induced lung hemorrhage in hamsters

The ability of FK706 to protect against human neutrophil elastase-induced lung hemorrhage was evaluated by using the method described in a previous report (Fletcher et al., 1990). Briefly, hamsters were anesthetized by intraperitoneal injection of 40 mg/kg of pentobarbital. 100 μl of saline (control) or saline containing human neutrophil elastase (50 μg/animal) was instilled intratracheally via a small incision in the ventral neck region by using a 250 μl syringe with a 1 cm length of small diameter tubing attached to a 27-gauge needle. The incisions were closed with surgical quick-set adhesive. Various doses of FK706 or α1-proteinase inhibitor in 100 μl of saline were administered intratracheally 5 min before enzyme injection. In the case of intravenous or oral administration, each 5 ml/kg of inhibitor was administered 3 or 30 min before enzyme injection, respectively. Control animals were given 100 μl of saline only before enzyme instillation. 3 h after enzyme injection, the animals were killed by CO_2 asphyxiation. The trachea was re-exposed and a 16-gauge needle was inserted and held in place with a surgical suture. The lungs were then lavaged using a single 2.5 ml aliquot of saline in a 2.5 ml syringe by gently expanding the lungs and then withdrawing the saline a total of three times, yielding a final volume of approximately 1.5 ml bronchoalveolar lavage fluid from each animal.

The recovered bronchoalveolar lavage fluid (250 μl) was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was aspirated off and 2 ml of distilled water was added to the pellet to cause cell disruption. This mixture was centrifuged at 1000 rpm for 5 min. Then, absorption by the supernatant was measured spectrophotometrically at 541 nm with a Hitachi U-3200 spectrophotometer and hemoglobin contents were expressed as OD 541 nm.

2.6. Human neutrophil elastase-induced paw edema in mice

Mice were injected subcutaneously in the right hind paw with 25 μl of saline or saline containing 0.8 mg/ml (20 μg/paw) of human neutrophil elastase and the left hind paw was injected with the same volume of saline. Various doses of FK706 in saline were administered subcutaneously 15 min before enzyme injection. At a given time paw thickness was measured with a slide caliper. Paw edema was expressed as the difference in paw thickness between the right and left hind paws.

2.7. Statistical analysis

In vitro IC_{50} values were calculated with an unweighted method for least-square fit of data (three values for each concentration). The statistical computer software package StatView version 4.02 was used for in vivo analysis using the data derived from 5 to 6 animals in each group. Data were evaluated using the paired *t*-test with significance at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. K_i value for human neutrophil elastase

Biphasic reaction progress curves, obtained during the inhibition of human neutrophil elastase by FK706 as illustrated in Fig. 2, indicated that FK706 is a slow-binding inhibitor of human neutrophil elastase. Reaction progress curves for compounds of this type are typically exponential followed by a positive linear slope, the reversible nature of FK706 being demonstrated by the positive linear, as opposed to a zero, slope. Progress curves for slow-binding inhibitors are described by the expression of Eq. (1), where A_0 and A_t are absorbance at time 0 and time t, respectively; v_0 and v_s are initial and steady state velocity, respectively and k is a pseudo-first order rate constant of inhibition:

$$A_t = v_s t + (v_0 - v_s)(1 - e^{-kt})/k + A_0 \quad (1)$$

K_i was calculated from Eq. (2) by using the values, v_0 and v_s from Eq. (1), where I and S are the concentration of inhibitor and substrate in the reaction mixture, respectively, K_M is a Michaelis constant for the substrate. In this analysis, K_M was constrained to 54 μM (Stein et al., 1987).

$$(v_0 - v_s)/v_s = I\{K_i(1 + S/K_M)\} \quad (2)$$

In our experiment FK706 appears to be a competitive inhibitor with a K_i of 4.2 nM and this small K_i suggests that the FK706-enzyme complex is hardly dissociated since the K_i value is calculated as the ratio of the dissociation constant of enzyme–inhibitor complex to its association rate constant in the slow-binding kinetics.

3.2. Determination of IC_{50} values for proteinases by using synthetic substrates

Inhibitory activities of FK706 against various proteinases evaluated using synthetic substrates were compared with the activity of α1-proteinase inhibitor as a control. IC_{50} value of FK706 against human neutrophil elastase was 83 nM and that of α1-proteinase inhibitor was 19 nM (Table 1). FK706 also inhibited mouse neutrophil elastase and porcine pancreatic elastase with respective IC_{50} values of 22 and 100 nM. FK706 has no inhibitory activity for human pancreatic α-chymotrypsin, human pancreatic trypsin and human neutrophil cathepsin G (IC_{50} values > 340 μM) under our assay conditions. On the other hand, α1-proteinase inhibitor inhibited various serine proteinases such as human and mouse neutrophil elastase ($IC_{50} = 19$ and 4.1 nM, respectively), porcine pancreatic elastase ($IC_{50} = 21$ nM), human pancreatic trypsin ($IC_{50} = 180$ nM), human pancreatic α-chymotrypsin ($IC_{50} = 5.7$ nM) and human leukocyte cathepsin G ($IC_{50} = 6.6$ nM). ONO-5046 also inhibited both human neutrophil elastase and porcine pancreatic elastase with respective IC_{50} values of 180 and 1300 nM. However, ONO-5046 was a much weaker inhibitor of human pancreatic trypsin ($IC_{50} > 340$ μM), human pancreatic α-chymotrypsin ($IC_{50} = 9.5$ μM) and human leukocyte cathepsin G ($IC_{50} = 7.2$ μM) as well as FK706. The potency of FK706 to inhibit human neutrophil elastase using a synthetic substrate was 4.4-fold less than that of α1-proteinase inhibitor and 2.2-fold greater than that of ONO-5046.

Table 1
Inhibitory activity of FK706 and α1-proteinase inhibitor against various proteinases using synthetic substrates

Proteinase	IC_{50} (nM)		
	FK706	α1-PI	ONO-5046
Human neutrophil elastase	83	19	180
Mouse neutrophil elastase	22	4.1	n.d.
Porcine pancreatic elastase	100	21	1300
Human pancreatic trypsin	> 340000	180	> 340000
Human pancreatic α-chymotrypsin	> 340000	5.7	9500
Human leukocyte cathepsin G	> 340000	6.5	7200

Inhibitory activities were determined using the following synthetic substrates (final concentration; 0.5 mM). Both human and mouse neutrophil elastase: methoxysuccinyl–Ala–Ala–Pro–Val– *p*-nitroanilide, porcine pancreatic elastase: *n*-succinyl–Ala–Ala–Ala– *p*-nitroanilide, both human pancreatic α-chymotrypsin and human neutrophil cathepsin G: methoxysuccinyl–Ala–Ala–Pro–Phe– *p*-nitroanilide and human pancreatic trypsin: *n*-benzoyl–Arg– *p*-nitroanilide. n.d.: not determined. α1-PI: α1-proteinase inhibitor.

3.3. Determination of IC_{50} values for human neutrophil elastase by using insoluble elastin

The ability of FK706 and α1-proteinase inhibitor to inhibit human neutrophil elastase hydrolysis of insoluble elastin was evaluated. The IC_{50} values of FK706 and α1-proteinase inhibitor to inhibit insoluble elastin degradation were 230 and 51 nM, respectively. The potency of FK706 was 4.5-fold less than that of α1-proteinase inhibitor when a natural substrate was used.

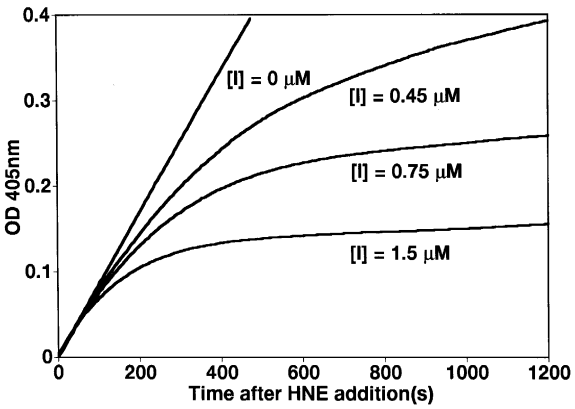

Fig. 2. Progress curves for the inhibition of human neutrophil elastase by FK706, for hydrolysis of the synthetic substrate, methoxysuccinyl–Ala–Ala–Pro–Val– *p*-nitroanilide. FK706 proved to be a slow-binding inhibitor with a $K_i = 4.2$ nM. HNE: human neutrophil elastase.

3.4. Human neutrophil elastase-induced lung hemorrhage in hamsters

The ability of FK706 to protect animals from human neutrophil elastase-induced lung hemorrhage was evaluated. Intratracheal instillation with 50 μg/animal of human neutrophil elastase induced a significant, severe, lung hemorrhage (OD 541 nm = 9.29 ± 1.15 to 19.00 ± 1.64 in each experiment (saline instillation; OD 541 nm = 0.26 ± 0.02 to 0.41 ± 0.07 in each experiment), mean ± S.E.M., $n = 6$, $P < 0.001$ compared to saline group in each experiment). Intratracheal treatment with FK706 at doses of 1, 10 and 100 μg/animal 3 min before human neutrophil elastase instillation produced a significant and dose-dependent inhibition of the hemorrhage, with an ED_{50} value of 2.4 μg/animal. Intratracheal treatment with FK706 at doses of 10 and 100 μg/animal almost restored the animals to the control level ($P < 0.001$) (Fig. 3A). Intravenous administration of FK706 at doses of 1, 10 and 100 mg/kg 3 min before enzyme instillation also protected against the hemorrhage in a dose-dependent manner, with an ED_{50} value of 36.5 mg/kg (Fig. 3B). Oral administration of FK706 30 min before enzyme instillation significantly ($P < 0.05$), but weakly suppressed the hemorrhage at doses of 1, 10 and 100 mg/kg (26, 28 and 31% inhibition, respectively) (Fig. 3C). On the other hand, intratracheal treatment with α1-proteinase inhibitor also

Table 2
Intratracheal pre- or post- treatment effect of FK706 on human neutrophil elastase-induced lung hemorrhage in hamsters

	Treatment time				
	−24 h	−2 h	−5 min	+5 min	+30 min
ED_{50} (μg/animal)	> 100	6.0	2.4	8.4	68.7

Hamsters ($n = 6$) were given FK706 intratracheally at the indicated time before or after 50 μg/animal of human neutrophil elastase instillation. Lung hemorrhage was examined 3 h after 50 μg/animal of human neutrophil elastase instillation.

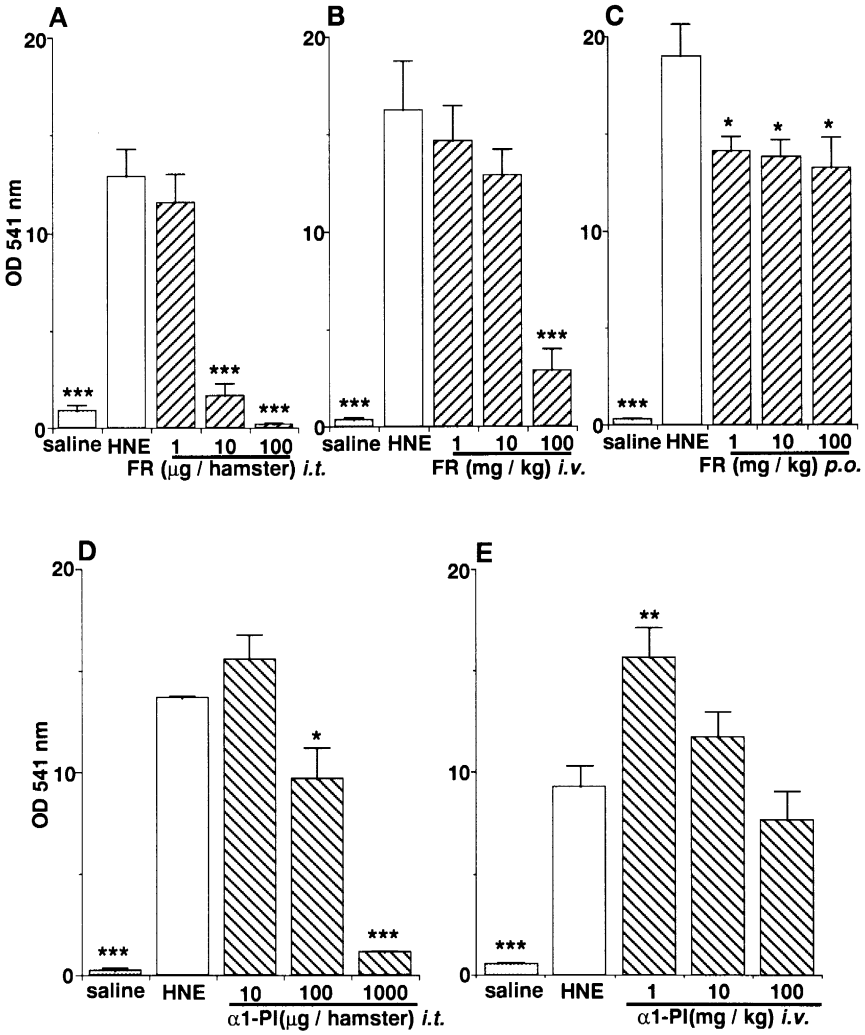

Fig. 3. Effects of FK706 and α1-proteinase inhibitor on human neutrophil elastase-induced lung hemorrhage in hamsters. FK706 was administered (A) intratracheally 5 min, (B) intravenously 3 min, (C) orally 30 min, before human neutrophil elastase (50 μg/animal) instillation. α1-proteinase inhibitor was (D) intratracheally 5 min, (E) intravenously 3 min, before human neutrophil elastase (50 μg/animal) instillation. Values are means ± S.E.M. for 6 hamsters. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus HNE. HNE: human neutrophil elastase. FR: FK706. α1-PI: α1-proteinase inhibitor.

protected against the hemorrhage in a dose-dependent manner, with an ED_{50} value of 228 μg/animal (Fig. 3D). The potency of α1-proteinase inhibitor given intratracheally to inhibit the hemorrhage was almost 10-fold less (maximum inhibition was 93.2% ($P < 0.001$) at a dose of 1000 μg/animal) than that of FK706. Intravenous administration of α1-proteinase inhibitor at doses of 1, 10 and 100 mg/kg was not effective (Fig. 3E).

We also examined the effect of FK706 administered intratracheally at various intervals before or after human neutrophil elastase instillation. The ED_{50} values increased with the interval between FK706 administration and human neutrophil elastase instillation. Both −2 h pre-treatment and +30 min post-treatment with FK706 were effective on lung hemorrhage in hamsters, with ED_{50} values of 6.0 and 68.7 μg/animal, respectively (Table 2).

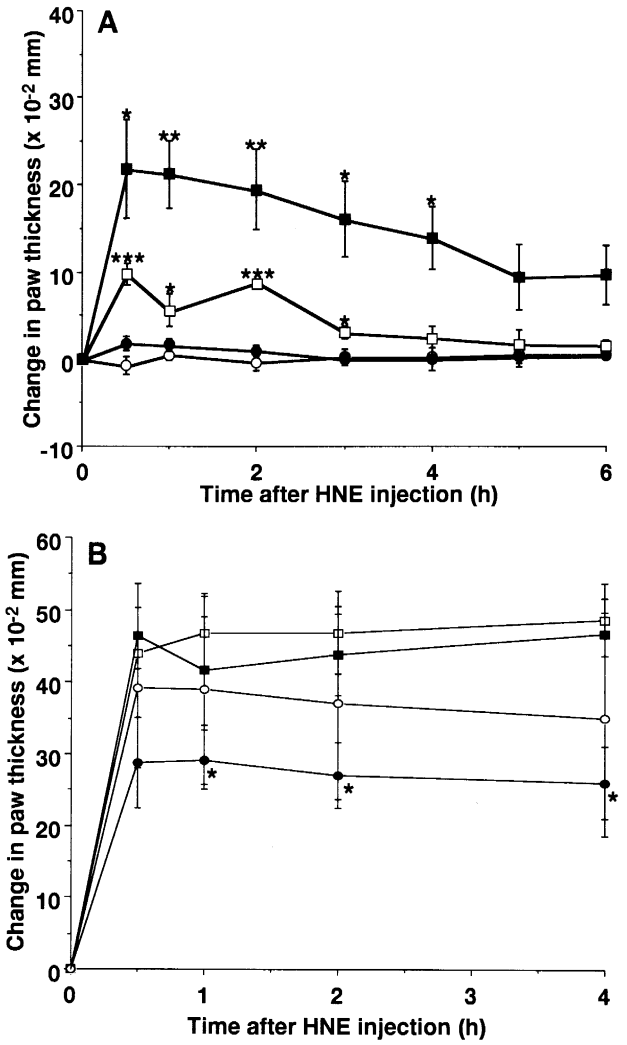

Fig. 4. Time-course of changes in paw edema induced by human neutrophil elastase in mice (A). Paw edema was expressed as the difference in paw thickness between right hind paw (human neutrophil elastase or saline) and left hind paw (saline). (○) Saline; (□) human neutrophil elastase, 1.5 μg/paw; (■) human neutrophil elastase, 5 μg/paw; (●) heat inactivated human neutrophil elastase, 5 μg/paw. Effect of FK706 on human neutrophil elastase induced paw edema in mice (B). FK706 was administered subcutaneously 15 min before 20 μg/paw of human neutrophil elastase injection. (■) Saline, FK706; (□) 10 mg/kg; (○) 32 mg/kg; (●) 100 mg/kg. Values are means ± S.E.M. for 5 to 6 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus saline (A) and * $P < 0.05$ versus HNE (B). HNE: human neutrophil elastase.

3.5. Human neutrophil elastase-induced paw edema in mice

Before evaluating the effect of FK706 on human neutrophil elastase-induced paw edema, we evaluated the paw edema induced by 1.5 and 5 μg/paw of enzyme. Human neutrophil elastase elicited dose-dependent increases in paw edema similarly to other irritants such as zymosan, carrageenan and bradykinin (Damas and Remacle-Volon, 1992). The increases in paw edema were 21.8 ± 5.7, 21.2 ± 3.8, 19.4 ± 4.6, 16.0 ± 4.4 and $13.8 \pm 3.6 \times 10^{-2}$ mm (means ± S.E.M., $n = 5$) at 0.5, 1, 2, 3 and 4 h after 5 μg/paw of human neutrophil injection (Fig. 4A).

The ability of FK706 to protect against the paw edema induced by 20 μg/paw of human neutrophil elastase was then evaluated because 5 μg/paw of human neutrophil was not severe enough to allow assessment of FK706. The increases in paw edema were 44.0 ± 2.3, 46.8 ± 5.1, 46.8 ± 5.8 and $48.6 \pm 5.1 \times 10^{-2}$ mm (means ± S.E.M., $n = 5$) at 0.5, 1, 2 and 4 h after 20 μg/paw of human neutrophil injection. Subcutaneous treatment with FK706 15 min prior to enzyme injection protected against paw edema in a dose-dependent manner (Fig. 4B). FK706 at a dose of 100 mg/kg significantly ($P < 0.05$) suppressed the increase in paw edema (29.2 ± 4.1 (38%), 27.0 ± 4.7 (42%) and 26.0 ± 5.1 (47%) $\times 10^{-2}$ mm (means ± S.E.M., $n = 5$) at 1, 2 and 4 h after enzyme injection, respectively).

4. Discussion

This study showed that FK706 is a potent, competitive and slow-binding inhibitor of human neutrophil elastase, with a K_i value of 4.2 nM. The IC_{50} values of FK706 for elastase-type endopeptidases such as human, mouse neutrophil elastase and porcine pancreatic elastase were 83, 22 and 100 nM, respectively. FK706 has no inhibitory activity (IC_{50} values > 340 μM) against other serine proteinases such as human pancreatic trypsin, human pancreatic α-chymotrypsin and human leukocyte cathepsin G under our assay conditions. FK706 was highly specific for elastase-type endopeptidases, since, at a concentration of 340 μM, FK706 had no effect on the various serine proteinases except elastases though we did not examine the inhibitory activity of FK706 for proteinase III. We cannot directly compare the proteinase selectivity of FK706 with that of other synthetic elastase inhibitors, because the latter was evaluated from the IC_{50} values rather than K_i values. However, the proteinase selectivity of FK706 using synthetic substrates was similar to that of other synthetic

inhibitors such as MR889 (Baici et al., 1990), ICI200, 880 (Williams et al., 1991b) and ONO-5046 (Kawabata et al., 1991) since our data for ONO-5046 on relative proteinase selectivity were very similar to those in the original report on ONO-5046. On the other hand, a natural endogenous proteinase inhibitor, α1-proteinase inhibitor, inhibited various serine proteinases such as human and mouse neutrophil elastase (IC_{50} = 19 and 4.1 nM, respectively), porcine pancreatic elastase (IC_{50} = 21 nM), human pancreatic trypsin (IC_{50} = 180 nM), human pancreatic α-chymotrypsin (IC_{50} = 5.7 nM) and human leukocyte cathepsin G (IC_{50} = 6.6 nM). According to the IC_{50} value, α1-proteinase inhibitor for human neutrophil elastase with methoxysuccinyl–Ala–Ala–Ala–Pro–Val–*p*-nitroanilide as a substrate was more potent than FK706. However, a more than 20-fold higher concentration of α1-proteinase inhibitor on a weight basis was needed to inhibit enzyme activity because it has a very high molecular mass (FK706: molecular mass = 0.59 kDa, IC_{50} = 0.049 μg/ml, α1-proteinase inhibitor: molecular mass = 54 kDa, IC_{50} = 1 μg/ml). Low molecular mass inhibitors which are therapeutically effective at low doses would have an advantage over high molecular mass inhibitors such as α1-proteinase inhibitor, secretory leukoproteinase inhibitor SLPI (secretory leukocyte protease inhibitor, 11.7 kDa) (Vogelmeier et al., 1991) and Eglin-C (8.1 kDa) (Schnebli et al., 1985).

Moreover, the relationship between pancreatic elastase and pancreatitis has been investigated and according to both early (Geokas et al., 1986) and more recent reports, pancreatic elastase inhibitors are effective on experimental acute pancreatitis (Fric et al., 1985). FK706 is expected to be effective in diseases in which neutrophil elastase and pancreatic elastase are concerned, because the inhibitory activity of FK706 against porcine pancreatic elastase is very high (IC_{50} = 100 nM), though we have not tested the inhibitory activity of FK706 against human pancreatic elastase. Its ability to protect against experimental pancreatitis is presently under investigation.

The inhibitory activity of FK706 was not confined to synthetic peptide substrates but extended, with similar magnitude, to the degradation by human neutrophil elastase of a macromolecular substrate such as elastin. The respective IC_{50} values of FK706 and α1-proteinase inhibitor to inhibit human neutrophil elastase hydrolysis of insoluble elastin were 230 nM and 51 nM. The relative difference between FK706 and α1-proteinase inhibitor in potency to inhibit human neutrophil elastase induced hydrolysis of natural substrate was very similar to the data on human neutrophil induced amidolysis by using the synthetic substrate in our assay condition.

As FK706 proved to inhibit the elastin degradation induced by human neutrophil elastase, the effects of FK706 on human neutrophil elastase-induced acute animal models were studied. The potency of FK706 given intratracheally to prevent the 50 μg/animal of human neutrophil elastase-induced lung hemorrhage was 80 times greater than that of α1-proteinase inhibitor, with respective ED_{50} values of 2.4 and 165 μg/animal. Furthermore, intratracheal treatment with FK706 appeared to suppress the ongoing lung hemorrhage induced in hamsters by human neutrophil elastase. This result suggests that FK706 may be an useful agent, not only for prevention but also for treatment. FK706 was also effective on human neutrophil elastase-induced lung hemorrhage, when given i.v., with an ED_{50} value of 36.5 mg/kg. Though oral administration of FK706 at doses of 1, 10 and 100 mg/kg significantly suppressed the lung hemorrhage, the efficacy was very weak. The fact that intravenous administration of FK706 is effective on elastase-induced lung hemorrhage is advantages for clinical use as an intravenous treatment for acute inflammatory diseases such as adult respiratory distress syndrome and septic shock. The i.v. administration of α1-proteinase inhibitor was not effective (ED_{50} > 100 mg/kg) in this model. Oral administration of α1-proteinase inhibitor was not tested because a large molecular glycoprotein α1-proteinase inhibitor is thought to be rapidly degraded by digestive juices. We developed the human neutrophil elastase-induced paw edema model in mice. Human neutrophil elastase also elicited paw edema as did other irritants such as zymosan, carrageenan and bradykinin. The paw edema in this model is thought to reflect increases in permeability of the peripheral capillaries. Vascular permeability abnormalities cause many diseases such as adult respiratory distress syndrome. Adult respiratory distress syndrome is thought to be related to increased activity of neutrophil elastase. Elastase increases capillary permeability, which may lead to lung edema. Subcutaneous treatment with FK706 was effective on human neutrophil elastase-induced paw edema in a dose dependent manner.

These data suggested that FK706 has advantages over α1-proteinase inhibitor and, moreover, that the substance has many desirable properties as a drug. For instance, FK706 is chemically stable and resistant to oxidative inactivation; it has neither antigenicity nor obvious toxic side-effects at high dose levels. In the experimental animal models, it was effective not only on intratracheal administration, but also on systemic administration.

In summary, FK706 is a potent, competitive and slow-binding inhibitor of human neutrophil elastase that inhibits both amidolytic activity (synthetic substrate) and elastolytic activity (insoluble elastin). FK706 protects animals against human neutrophil elastase-induced lung hemorrhage when given either intratracheally or intravenously, and against human neutrophil elastase-induced paw edema when given subcutaneously. The current results indicate that FK706 will be a useful agent for studying the pathogenic role of human neutrophil elastase in inflammatory diseases such as emphysema, adult respiratory distress syndrome, septic shock, cystic fibrosis and rheumatoid arthritis, given either by systemic administration or by inhalation. Furthermore, FK706 will also be a useful tool

for investigating other elastase-related diseases such as pancreatitis. We are presently studying the effects of FK706 on animal models of emphysema and pancreatitis.

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