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Biochemical and pharmacological characterization of FR134043, a novel elastase inhibitor

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Abstract

FR134043, disodium(*Z*,1*S*,15*S*,18*S*,24*S*,27*R*,29*S*,34*S*,37*R*)-29-benzyl-21-ethylidene-27-hydroxy-15-isobutyrylamino-34-isopropyl-31,37-dimethyl-10,16,19,22,30,32,35,38-octaoxo-36-oxa-9,11,17,20,23,28,31,33-octaazatetracyclo[16.13.6.1$^{24,\ 28}$.0$^{3,\ 8}$]octatriconta-3,5,7-trien-5,6-diyl disulfate, is a water-soluble inhibitor of human neutrophil elastase with a molecular mass of 1166.15 Da. FR134043 demonstrated a characteristic competitive inhibition of human neutrophil elastase with a K_i of 8 nM. In studies using synthetic substrates, FR134043 inhibited both neutrophil elastase activity and porcine pancreatic elastase activity with IC_{50} values of 35 nM and 49 nM respectively. FR134043 also inhibited hydrolysis of bovine neck ligament elastin by human neutrophil elastase with an IC_{50} value of 210 nM. In in vivo experiments, FR134043 protected animals against human neutrophil elastase (50 μg/animal)-induced lung hemorrhage in hamsters with an ED_{50} value of 3.1 μg/animal for intratracheal administration and 5.0 mg/kg for intravenous administration. Subcutaneous treatment with FR134043 significantly suppressed human neutrophil elastase (20 μg/paw)-induced paw edema in mice with an ED50 value of 3.3 mg/kg when evaluated 4 h after elastase injection. The potency of FR134043 given intratracheally to protect against porcine pancreatic elastase (100 μg/animal)-induced emphysema in hamsters was relatively low (Quasi-static lung compliance; ED_{50} = 1590 μg/animal) compared to that in acute animal models. FR134043 (10 mg/kg per h i.v. infusion) significantly improved lipopolysaccharide (0.25 mg/kg per h i.v. infusion)-induced thrombocytopenia and some coagulation parameters in rats. These results suggest that systemic administration of FR134043 would be advantageous over intratracheal administration of FR134043 for the treatment of adult respiratory distress syndrome, septic shock and pulmonary emphysema and other pathophysiologic conditions in which elastases are thought to be involved. © 1998 Elsevier Science B.V.

Keywords: FR134043; Elastase inhibitor; Elastase; Adult respiratory distress syndrome; Disseminated intravascular coagulation; Septic shock; Pulmonary emphysema; Hemorrhage; Edema

1. Introduction

Elastases are members 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 (Groutas, 1987; Janoff, 1985), 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, 1978), and other inflammatory states (Adeyemi et al., 1985; Fric et al., 1985).

Extracellular human neutrophil elastase released from 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–antielastase imbalance caused by oxidative inactivation or a genetic deficiency of α1-proteinase inhibitor

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(Carp et al., 1982; Gadek and Pacht, 1990; Cox and Levison, 1988). Indeed, individuals deficient in α1-proteinase inhibitor (Pizz 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 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 synthetic low-molecular-weight inhibitors of human neutrophil elastase have been described and reviewed (Williams et al., 1991a,b; Fletcher et al., 1990; Kawabata et al., 1991).

We recently isolated a novel elastase inhibitor, FR901277, from the culture filtrate of strains *of Streptomyces resistomycificus* No. 7622 (Fujie et al., 1993). FR134043, disodium(*Z*,1*S*,15*S*,18*S*,24*S*,27*R*,29*S*,34*S*,37*R*)-29-benzyl-21-ethylidene-27-hydroxy-15-isobutyrylamino-34-isopropyl-31,37-dimethyl-10,16,19,22,30,32,35,38-octaoxo-36-oxa-9,11,17,20,23,28,31,33-octaazatetracyclo[16.13.6.1$^{24,\ 28}$.0$^{3,\ 8}$]octatriconta-3,5,7-trien-5,6-diyl disulfate, $C_{47}H_{61}N_9Na_2O_{19}S_2$, is a water-soluble derivative of FR901277 and consists of a cyclic peptide lactone with a molecular mass of 1166.15 Da (Fig. 1). The solubility of FR134043 is greater than 420 mg/ml at 37°C, while that of FR901277 is 0.39 mg/ml at 37°C.

This report describes the in vitro profile of FR134043 and its inhibitory effects on the actions of elastase and lipopolysaccharide in various animal models.

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 (Green et al., 1991; Fletcher et al., 1990; Skiles et al., 1984). Porcine pancreatic elastase (EC 3.4.21.36) (Type IV, 69 units/mg protein using elastin as a substrate), bovine pancreatic α-chymotrypsin (EC 3.4.21.1) (54 units/mg protein using *n*-benzoyl-Tyr-ethyl-ester (BTEE) as a substrate), bovine pancreatic trypsin (EC 3.4.21.4) (10,700 units/mg protein using *n*-benzoyl-Arg-ethyl-ester (BAEE) as a substrate), methoxysuccinyl-Ala–Ala-Pro-Val-*p*-nitroanilide, *n*-succinyl-Ala–Ala–Ala-*p*-nitroanilide, methoxysuccinyl-Ala–Ala-Pro-Met-*p*-nitroanilide, *n*-benzoyl-Arg-*p*-nitroanilide and α1-proteinase inhibitor (2.8 mg inhibits 1.0 mg of trypsin with an activity of 10 000 BAEE units/mg protein, 3.5 mg inhibits 1.0 mg of α-chymotrypsin with an activity of 40–50 BAEE units/mg protein), and lipopolysaccharide (from *Escherichia coli* No. 011 B4) were purchased from Sigma Chemicals, St. Louis, MO, USA. Porcine pancreatic elastase (EC 3.4.21.36) (135 units/mg protein using elastin as a substrate) for induction of experimental emphysema was purchased from Elastin Products, Pacific, MO, USA. Archronium chloride (Dialferin®) was purchased from Japan Roche, Tokyo, Japan. Other chemicals were of reagent grade.

Female golden hamsters (Mesocricetus auratus) aged five weeks, weighing approximately 80 g, were obtained from Charles River Wilmington, MA, USA. Male golden Syrian hamsters, weighing approximately 120 g, and male Wistar rats aged 6–7 weeks were obtained from Japan

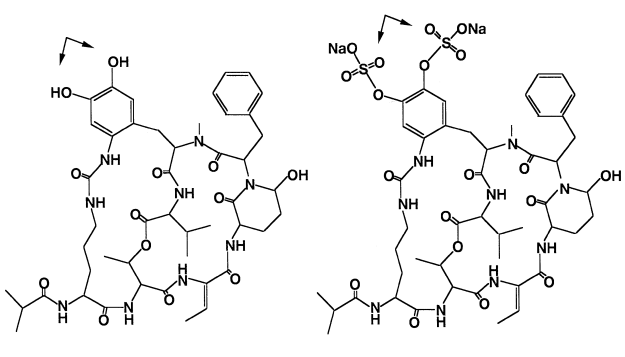

Fig. 1. Chemical structure of FR134043, disodium (*Z*,1*S*,15*S*,18*S*,24*S*,27*R*,29*S*,34*S*,37*R*)-29-benzyl-21-ethylidene-27-hydroxy-15-isobutyrylamino-34-isopropyl-31,37-dimethyl-10,16,19,22,30,32,35,38-octaoxo-36-oxa-9,11,17,20,23,28,31,33-octaazatetracyclo [16.13.6.1$^{24,\ 28}$.0$^{3,\ 8}$]octatriconta-3,5,7-trien-5,6-diyl disulfate, $C_{47}H_{61}N_9Na_2O_{19}S_2$. The arrows show the structural differences between FR901277 and FR134043.

SLC, Shizuoka, Japan, and male C57BL mice aged 6 weeks were obtained from Clea Japan.

Ninety-six-well microtiter plates (MS3496FE) were purchased from Sumitomo Bakelite, Tokyo, Japan. All parts of a constant volume, whole-body plethysmograph were purchased from Nihon Kohden, 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 (Williams et al., 1991a). Briefly, the synthetic substrate methoxysuccinyl-Ala–Ala-Pro-Val-*p*-nitroanilide was hydrolyzed by human neutrophil elastase to *p*-nitroaniline at 25°C. Hydrolysis was continuously measured spectrophotometrically by monitoring absorbance changes at 410 nm with a Hitachi U-3200 spectrophotometer. The substrate was dissolved in 100% DMSO (dimethyl sulfoxide). Two milliliters of reaction mixture contained 1850 μl of 0.1 M HEPES, 0.5 M NaCl pH 7.5, 50 μl of substrate, and 50 μl of inhibitor dissolved in the above buffer (final DMSO concentration; 2.5%). The reaction was started by addition of 50 μl of 200 nM of enzyme solution (final enzyme concentration; 5 nM). Activity against the substrate was determined by the increase in optical density at 410 nm over 5 min. A K_i apparent was obtained from the intersection of the lines determined by a non-weighted least-square fit of the data.

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

IC_{50} values for various proteinases were determined using a minor modification of the method described previously (Bonney et al., 1989). Briefly, 100 μl of incubation mixture containing 50 μl of inhibitor dissolved in 6.4% DMSO, 0.5 mM 25 μl of substrate dissolved in 4% DMSO (final DMSO concentration; 4.2%), and 25 μl of enzyme in (added last) in 0.1 M HEPES 0.5 M NaCl, pH 7.5 was incubated in the wells of 96-well microtiter plates at room temperature for 30 min. After incubation, the release of *p*-nitroaniline was assayed at OD 415 nm with a BIO-RAD Model 3550 Microplate Reader. Inhibitory activities of each inhibitor against various proteinases were determined from the increases in optical density at 415 nm. Human neutrophil elastase at a final concentration of 50 nM was assayed using methoxysuccinyl-Ala–Ala-Pro-Val-*p*-nitroanilide as a substrate. Porcine pancreatic elastase at a final concentration of 200 nM was assayed using *n*-succinyl-Ala–Ala–Ala-*p*-nitroanilide (Fujimoto et al., 1980) as a substrate, α-chymotrypsin at a final concentration of 640 nM was assayed using methoxysuccinyl-Ala–Ala-Pro-Met-*p*-nitroanilide (Nakajima and Powers, 1979) as a substrate, and trypsin at a final concentration of 64 nM was assayed using *n*-benzoyl-Arg-*p*-nitroanilide (Lee et al., 1987) as a substrate.

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

The ability of FR134043 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 described in a 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 = 133 nM) were incubated with various concentrations of FR134043 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 FR134043 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, female hamsters were anesthetized by intraperitoneal injection of 40 mg/kg of pentobarbital. One hundred microliters 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 FR134043 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 min or 30 min before enzyme injection, respectively. Control animals were given 100 μl of saline only before enzyme instillation. Three hours 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 with a single 2.5-ml volume 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, the absorption of the

supernatant was measured spectrophotometrically at 541 nm with a Hitachi U-3200 spectrophotometer, and the hemoglobin content was 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 FR134043 in saline were administered subcutaneously in the back of mice 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. Porcine pancreatic elastase-induced emphysema in hamsters

Male hamsters were anesthetized intraperitoneally with pentobarbital. Various doses of either FR134043 or α1-proteinase inhibitor in 0.2 ml of saline were administered intratracheally by the transoral route 5 min before instillation of 100 μg of porcine pancreatic elastase in 0.2 ml of saline. Two weeks after enzyme instillation, the hamsters were anesthetized with pentobarbital and pulmonary function was measured in supine hamsters using the constant volume, whole-body plethysmograph. Quasi-static deflation pressure–volume curves were obtained by intraperitoneally administering 5 mg/kg of archronium chloride to suppress spontaneous breathing, inflating the lungs to a transpulmonary pressure of 30 cmH_2O, deflating them to a transpulmonary pressure of 0 cmH_2O and aspirating them to a transpulmonary pressure of -20 cmH_2O. This was done by changing the pressure in the animal box by means of an automatic pressure controller. Quasi-static lung compliance was defined as the slope of the steep portion of the deflation pressure–volume curve in the mid-volume range. Vital capacity was defined as the difference in lung volume between total lung capacity at a transpulmonary pressure of 25 cmH_2O and residual volume at a transpulmonary pressure of -20 cmH_2O (Koo et al., 1976).

2.8. Experimental disseminated intravascular coagulation in rats

Experimental disseminated intravascular coagulation was induced in rats according to the method of Imura et al. (1986) with slight modifications. Male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and right femoral veins were cannulated for infusion of lipopolysaccharide and FR134043. Lipopolysaccharide at 0.25 mg/kg per h was infused at a flow rate of 0.6 ml/h for 4 h. Blood samples were withdrawn from the abdominal aorta 4 h after lipopolysaccharide infusion to determine coagulation parameters in rats with disseminated intravascular coagulation. The samples used to determine the coagulation parameters were anticoagulated with 3.8% sodium citrate (1/10 volume). The coagulation parameters estimated were; platelet counts, blood leukocyte counts, prothrombin time, activated partial thromboplastin time, fibrinogen and fibrinogen/fibrin degradation products. Platelets and leukocytes were counted using an automatic counter (Sysmex PL 110, Toaiyodensi, Japan). Fibrinogen/fibrin degradation products were measured with a commercial kit (Teikokuzoki Pharmaceutical, Japan), which utilizes a latex agglutination test. Prothrombin time, activated partial thromboplastin time and fibrinogen were determined using an autoalalyzar (Amelung-Coagulometer KC10A, Amelung, Germany).

2.9. Statistical evaluation of data

In vitro IC_{50} values were calculated by using a non-weighted least-square fit of the data (three data in each concentration). The statistical computer software package StatView version 4.02 was used for in vivo analysis, using the data derived from 6 to 8 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

Dixon plot analysis showed that FR134043 was a competitive inhibitor of human neutrophil elastase with a K_i value of 8 nM (Fig. 2).

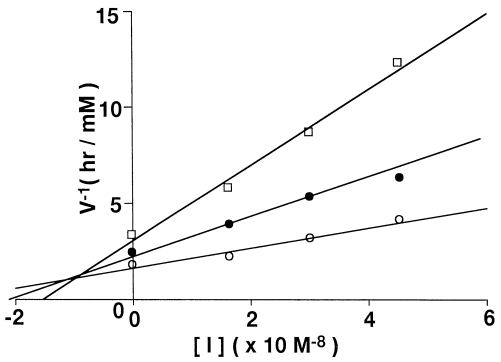

Fig. 2. Dixon plot for inhibition by FR134043 of human neutrophil elastase hydrolysis of the synthetic substrate, methoxysuccinyl-Ala–Ala-Pro-Val-*p*-nitroanilide. [S] = methoxysuccinyl-Ala–Ala-Pro-Val-*p*-nitroanilide concentration; ○, [S] = 500 μM; ●, [S] = 200 μM; □, [S] = 100 μM. FR134043 appears to be a competitive inhibitor with a $K_i = 8$ nM.

Table 1
Inhibitory activity of FR134043 and α1-proteinase inhibitor against various proteinases

Proteinase	IC_{50} (nM)	
	FR134043	α1-proteinase inhibitor
Human neutrophil elastase	35	64
Human neutrophil elastase (natural substrate)	210	51
Porcine pancreatic elastase	49	160
Bovine pancreatic trypsin	200	90
Bovine pancreatic α-chymotrypsin	1800	620

Inhibitory activities were determined using the following synthetic and natural substrates. Human neutrophil elastase; methoxysuccinyl-Ala–Ala-Pro-Val-*p*-nitroanilide and bovine neck ligament elastin (natural substrate), porcine pancreatic elastase; *n*-succinyl-Ala–Ala–Ala-*p*-nitroanilide, bovine pancreatic trypsin; *n*-benzoyl-Arg-*p*-nitroanilide, and bovine pancreatic α-chymotrypsin; methoxysuccinyl-Ala–Ala-Pro-Met-*p*-nitroanilide.

3.2. Inhibition of proteinases against synthetic substrates

Inhibitory activities of FR134043 and α1-proteinase inhibitor against various proteinases were evaluated using synthetic substrates. The IC_{50} value of FR134043 against human neutrophil elastase was 35 nM, and that of α1-proteinase inhibitor was 64 nM (Table 1). The IC_{50} value of FR134043 against porcine pancreatic elastase was 49 nM, and that of α1-proteinase inhibitor was 160 nM. FR134043 also inhibited other serine proteinases such as α-chymotrypsin and trypsin with an IC_{50} value of 18 000 nM and 200 nM, respectively. α1-Proteinase inhibitor inhibited various proteinases such as human neutrophil elastase, porcine pancreatic elastase, bovine pancreatic α-chymotrypsin and trypsin with IC50 values in the order of 64 to 620 nM under our assay conditions.

3.3. Inhibition of human neutrophil elastase against insoluble elastin

The IC_{50} values of FR134043 and α1-proteinase inhibitor to inhibit insoluble elastin degradation were 210 nM and 51 nM, respectively (Table 1). The potency of FR134043 was 4-fold less than that of α1-proteinase inhibitor when a natural substrate was used.

3.4. Human neutrophil elastase-induced lung hemorrhage in hamsters

The ability of FR134043 to protect animals from human neutrophil elastase-induced lung hemorrhage was evaluated. Intratracheal instillation with 50 μg/animal of human neutrophil elastase induced severe lung hemorrhage (OD 541 nm = 9.29 ± 1.15 to 23.83 ± 3.26 in each experiment (saline instillation; OD 541 nm = 0.22 ± 0.04 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 FR134043 at doses of 1, 10, 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 3.1 μg/animal (2.7 nmol/animal; Fig. 3A). Intratracheal treatment with FR134043 at a dose of 100 μg/animal provided almost complete protection against hemorrhage ($P < 0.001$). Intravenous administration of FR134043 at doses of 1, 10, 100 mg/kg 3 min before enzyme instillation also protected against the hemorrhage in a dose-dependent manner with an ED_{50} value of 5.0 mg/kg (4.3 μmol/kg; Fig. 3C). Intratracheal treatment with α1-proteinase inhibitor also protected against the hemorrhage in a dose-dependent manner with an ED_{50} value of 228 μg/animal (4.2 nmol/animal; Fig. 3B). Intravenous treatment with α1-proteinase inhibitor at doses of 1, 10, 100 mg/kg was not effective (Fig. 3D). We also examined the effect of FR134043 intratracheally administered at various intervals before or after human neutrophil elastase instillation. ED_{50} values increased with the interval between FR134043 administration and human neutrophil elastase instillation. Both −2 h pre-treatment and +30 min post-treatment with FR134043 were effective in protecting against lung hemorrhage in hamsters, with ED50 values with 15.2 μg/animal (13.0 nmol/animal) and 4.2 μg/animal (3.6 nmol/animal), respectively.

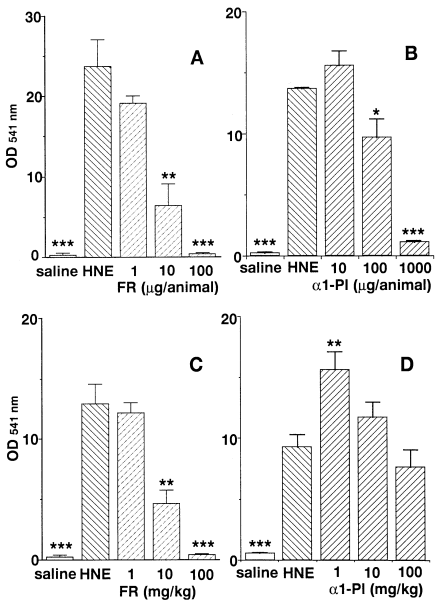

Fig. 3. Effects of FR134043 and α1-proteinase inhibitor on human neutrophil elastase-induced lung hemorrhage in hamsters. FR134043 and α1-proteinase inhibitor were administered intratracheally 5 min (A, B) or intravenously 3 min (C, D) before human neutrophil elastase (50 μg/animal) instillation. Values are means ± S.E.M. for 6 hamsters. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. human neutrophil elastase. HNE; human neutrophil elastase. FR; FR134043, α1-PI; α1-proteinase inhibitor.

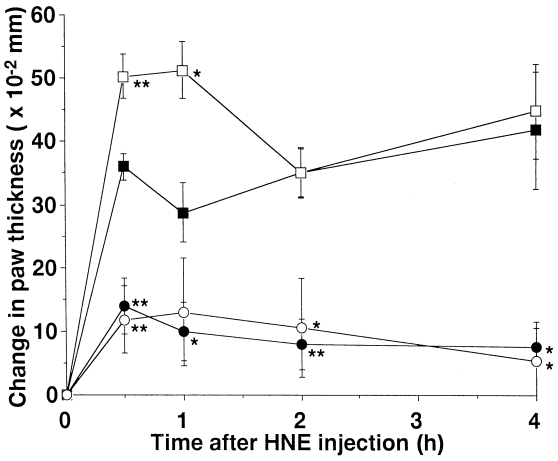

Fig. 4. Effect of FR134043 on human neutrophil elastase-induced paw edema in mice. FR134043 was administered subcutaneously 15 min before injection of 20 μg/paw of human neutrophil elastase. ■; saline, FR134043, □; 10 mg/kg, ○; 32 mg/kg, and ●; 100 mg/kg. Values are means ± S.E.M. for 6 mice. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs. human neutrophil elastase.

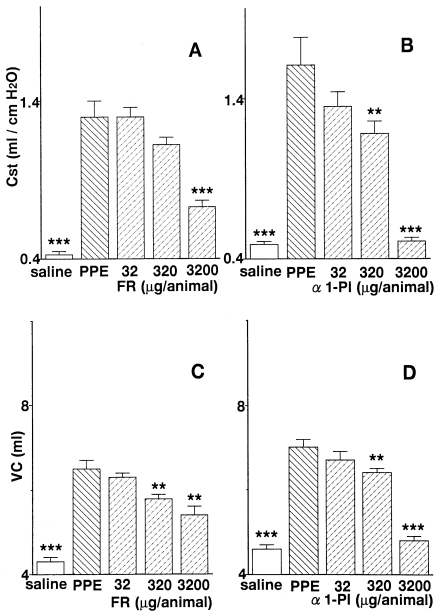

Fig. 5. Effect of FR134043 and α1-proteinase inhibitor on increases in quasi-static lung compliance (A, B) and vital capacity (C, D) induced by intratracheal instillation of 100 μg/animal of porcine pancreatic elastase in hamsters. Respiratory variables were measured 2 weeks after intratracheal instillation of porcine pancreatic elastase −5 min before instillation of either FR134043 or α1-proteinase inhibitor. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. porcine pancreatic elastase. Cst; quasi-static lung compliance, VC; vital capacity PPE; porcine pancreatic elastase, FR; FR134043, α1-PI; α1-proteinase inhibitor.

3.5. Human neutrophil elastase-induced paw edema in mice

The ability of FR134043 to protect against paw edema induced by 20 μg/paw of human neutrophil was evaluated. The increase in paw edema was 36.0 ± 2.1, 28.8 ± 4.7, 35.0 ± 3.8, and $41.8 \pm 9.3 \times 10^{-2}$ 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 FR134043 15 min prior to enzyme injection protected against paw edema in a dose-dependent manner (Fig. 4). FR134043 at a dose of 100 mg/kg significantly suppressed the increase in paw edema. (11.8 ± 5.3 (67%), 13.0 ± 8.5 (55%), 10.5 ± 7.8 (70%), and 5.3 ± 6.3 (87%) $\times 10^{-2}$ mm, (means ± S.E.M., n = 5) at 0.5, 1, 2, and 4 h after enzyme injection, respectively) The ED50 values of FR134043 in this model were 6.6 mg/kg (5.7 μmol/kg) at 0.5 h, 6.5 mg/kg (5.6 μmol/kg) at 1 h, 3.2 mg/kg (2.7 μmol/kg) at 2 h, and 3.3 mg/kg (2.8 μmol/kg) at 4 h, respectively.

3.6. Porcine pancreatic elastase-induced emphysema in hamsters

The ability of FR134043 to protect against porcine pancreatic elastase-induced emphysema in hamsters was evaluated by measuring pulmonary function. Intratracheal treatment with FR134043 at a dose of 3200 μg/animal 5 min before the intratracheal instillation of 100 μg/animal of porcine pancreatic elastase significantly protected against porcine pancreatic elastase-induced emphysematous changes in both quasi-static lung compliance ($P < 0.001$) and vital capacity. ($P < 0.05$; Fig. 5A, C) ED50 values were 1590 μg/animal (1.4 μmol/animal) and 2965 μg/animal (2.5 μmol/animal) for quasi-static lung compliance and vital capacity, respectively, and those of α1-proteinase inhibitor were 587 μg/animal (10.9 nmol/animal) and 924 μg/animal (17.1 nmol/animal), respectively (Fig. 5B, D).

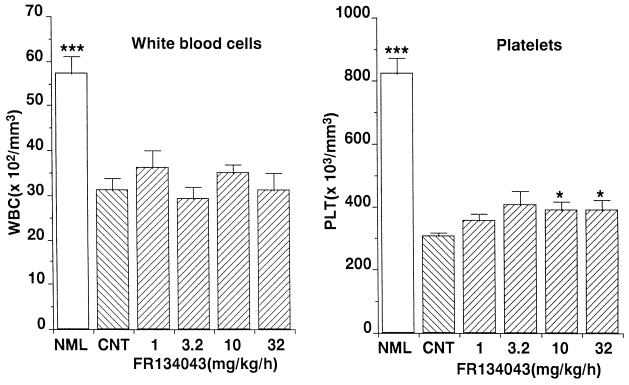

Fig. 6. Effect of FR134043 on leukopenia and thrombocytopenia caused by lipopolysaccharide infusion in rats. Lipopolysaccharide at 0.25 mg/kg/ per h was infused continuously for 4 h in rats simultaneously treated or not with FR134043. Blood samples were collected at the end of the infusion. The results are expressed as means + S.E.M. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control. NML; Normal (saline treated), CNT; control (lipopolysaccharide treated).

Table 2
Effects of FR134043 on parameters of intravascular coagulation induced by lipopolysaccharide in rats

	n (s)	PT (s)	APTT (mg/dl)	Fibrinogen (μg/ml)	FDP (μg/ml)
Normal	6	5.3 ± 0.4[a]	20.3 ± 0.3[a]	231 ± 9[a]	0.1 ± 0.1[a]
Control	5	21.8 ± 0.7	50.3 ± 3.9	40 ± 6	3.8 ± 0.4
FR134043 (mg/kg per h)					
1	6	20.5 ± 0.7	42.9 ± 3.4	52 ± 9	3.3 ± 0.3
3.2	6	20.5 ± 0.7	40.0 ± 1.8[b]	62 ± 13	2.8 ± 0.5
10	6	19.6 ± 0.8	39.1 ± 1.6[b]	49 ± 7	2.5 ± 0.4[b]
32	6	20.4 ± 1.3	42.5 ± 2.3	48 ± 8	2.5 ± 0.5

Lipopolysaccharide was infused for 4 h with or without simultaneous treatment with FR134043. Blood samples were collected at the end of the infusion. PT, APTT and FDP represent prothrombin time, activated partial thromboplastin time and fibrinogen/fibrin degradation products, respectively. The results are expressed means ± S.E.M.
[a] $P < 0.001$ and [b] $P < 0.05$; significantly different from the control.

3.7. Experimental disseminated intravascular coagulation model in rats

The ability of FR134043 to protect against experimental disseminated intravascular coagulation in rats was evaluated by measuring coagulation parameters. The treatment with FR134043 had no effect on the leukopenia at 4 h after lipopolysaccharide infusion. In contrast, the lipopolysaccharide-induced reduction in blood platelet count was slightly but significantly inhibited by FR134043 at 10 and 32 mg/kg per h (Fig. 6). As shown in Table 2, in the control group, prothrombin time and activated partial thromboplastin time were significantly prolonged from 5.3 to 21.8 s ($P < 0.001$) and 20.3 to 50.3 s ($P < 0.001$), respectively. The level of fibrinogen was pronouncedly decreased from 231 to 40 mg/dl ($P < 0.001$) and that of fibrinogen/fibrin degradation products was increased from 0.1 to 3.8 μg/ml ($P < 0.001$). Treatment with FR134043 at doses of 3.2 and 10 mg/kg per h (2.7 and 8.6 μmol/kg per h) significantly improved activated partial thromboplastin time ($P < 0.05$), and treatment with FR134043 at a dose of 10 mg/kg per h (8.6 μmol/kg per h) significantly improved fibrinogen/fibrin degradation time ($P < 0.05$).

4. Discussion

We synthesized FR134043, a water-soluble derivative of FR901277, which is a human neutrophil elastase inhibitor discovered from culture filtrate of *Streptomyces resistomicificus* No. 7622. The improvement in solubility did not affect the basic characteristics of FR134043 compared to those of the original material, FR901277 (Fujie et al., 1993). However, this allowed us to examine the effects of FR134043 in various animal models, because FR134043 can be given in solution.

This study showed that FR134043 is a competitive inhibitor of human neutrophil elastase with a K_i value of 8 nM. In study with synthetic substrates, FR134043 inhibited both human neutrophil and porcine pancreatic elastase with IC_{50} values of 35 nM and 49 nM respectively. Though FR134043 inhibited other serine proteinases such as α-chymotrypsin ($IC_{50} = 18\,000$ nM) and trypsin ($IC_{50} = 200$ nM), the potency was less than that for elastase-type endopeptidase. Natural endogenous proteinase inhibitor, α1-proteinase inhibitor, inhibited various serine proteinases such as human neutrophil elastase, porcine pancreatic elastase, α-chymotrypsin and trypsin with almost similar potency (IC_{50} values = 64–620 nM) under our assay conditions. Elastase inhibitors can be classified into three categories. The first category, neutrophil elastase specific inhibitors such as ICI200,880 (Williams et al., 1991b), and SC39026 (Nakao et al., 1987), the second category, both neutrophil and pancreatic elastase specific inhibitors such as MR889 (Baici et al., 1990), ONO5046 (Kawabata et al., 1991), and FK706 (Shinguh et al., 1997), and the last category, inhibitors that are relatively specific for elastases, but which inhibit various serine proteinase broadly such as α1-proteinase inhibitor and FR134043. The inhibitory activity of FR134043 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 IC_{50} values of FR134043 and α1-proteinase inhibitor to inhibit the hydrolysis by human neutrophil elastase of insoluble elastin were 210 nM and 51 nM respectively. According to the IC_{50} value, the potency of FR134043 for human neutrophil elastase with insoluble elastin as a substrate was 4-fold less than that of α1-proteinase inhibitor, though the potency of FR134043 was 2-fold greater than that of α1-proteinase inhibitor when a synthetic substrate for human neutrophil elastase, methoxysuccinyl-Ala–Ala-Pro-Val-*p*-nitroanilide, was used.

As FR134043 inhibited the degradation of the natural substrate, insoluble elastin, by elastases, the effects of FR134043 on human neutrophil elastase-induced hemor-

rhage was studied. The ability of FR134043 given intratracheally to prevent lung hemorrhage was very potent (ED50 = 3.1 μg/animal). Furthermore, FR134043 was also effective against human neutrophil elastase-induced lung hemorrhage in low doses administered by the intravenous route (ED50 = 5.0 mg/kg). Most of the effects of other low-molecular-weight inhibitors on lung hemorrhage have been evaluated following intratracheal inhibitor administration (Fletcher et al., 1990; Kawabata et al., 1991). The observation that intravenous administration of FR134043 was effective against elastase-induced lung hemorrhage is advantageous for its clinical use, especially as intravenous administration of α1-proteinase inhibitor was not effective in this model. Intravenous treatment with high doses of α1-proteinase inhibitor is not physiological and most of the α1-proteinase inhibitor is thought to be oxidized and inactivated during its circulation.

Human neutrophil elastase elicited paw edema, as do other irritants such as zymosan, carrageenan and bradykinin (Damas and Remacle-Volon, 1992). The paw edema of this model is thought to reflect an increase in the 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 an increased activity of neutrophil elastase. Elastase increases capillary permeability, which may lead to lung edema. Subcutaneous treatment with FR134043 at low doses was effective against human neutrophil elastase-induced paw edema in a dose-dependent manner.

We examined the ability of FR134043 to protect against porcine pancreatic elastase-induced emphysema in hamsters. The mechanism by which porcine pancreatic elastase induces emphysema is still controversial. Some investigators have suggested that instillation of porcine pancreatic elastase in the lung causes lung hemorrhage and lung inflammation and secondly triggers the release of endogenous neutrophil elastases (Lai and Diamond, 1990), and that porcine pancreatic elastase causes an increase in alveolar macrophages, a source of elastolytic enzymes (Finlay et al., 1996). Other investigators have suggested that porcine pancreatic elastase directly degrades elastin in the alveolar wall (Morris et al., 1986). The instillation of a large amount of human neutrophil elastase in the lungs of rodents is not physiological and many hamsters died due to severe lung hemorrhage. However, the surviving hamsters were relatively active, and emphysematous lung changes were very slight, as examined by the measurement of pulmonary function. The potency of FR134043 given intratracheally to protect against porcine pancreatic elastase-induced emphysema in hamsters (ED_{50} = 1590 μg/animal on quasi-static lung compliance) was very low (more than 660-fold less than its potency to inhibit human neutrophil elastase-induced lung hemorrhage in hamsters (ED_{50} = 2.4 μg/animal) in our experiment. These results supported the findings of Stone et al. (1990), who proposed that assessment of the ability of elastase inhibitors to suppress pulmonary hemorrhage is not predictive of their ability to ameliorate emphysema. The reason for the diminished effectiveness of FR134043 against elastase-induced emphysema is not due to its inability to inhibit rodent elastases, because FR134043 inhibited the elastase of hamsters as well as that of rats and mice with similar potency (data not shown). The difference between the potency against hemorrhage and that against emphysema is thought to be due to half-lives of instilled enzyme and inhibitor in the lung, that is, the lack of stability and continuity of FR134043 in the lung. In order to investigate the stability of FR134043 in organs, we studied the half-life of FR134043 in various tissue homogenates. The in vitro half-life of FR134043 in rat plasma, serum, and in 30% rat liver homogenate was more than 24 h, but in 10% rat lung homogenate and in 10% rat intestine homogenates its half-life was 3.3 h and 11.6 h, respectively, while the half-lives of both human neutrophil elastase and porcine pancreatic elastase in hamster lung are reported to be 1 h (Lucey et al., 1988). The half-life of FR134043 in the lung in vitro was 3-fold longer than that of the elastases examined in vivo. However, the clearance of FR134043 from the lung may be relatively rapid, although we did not examine the half-life of FR134043 in the intact lung.

Uchida et al. (1995) reported that lipopolysaccharide-induced pulmonary vascular injury was significantly attenuated in rats treated with ONO5046, but ONO5046 did not inhibit the increase in serum fibrinogen/fibrin degradation products. In contrast, continuous *i.v.* infusion of FR134043 attenuated lipopolysaccharide-induced thrombocytepenia and the parameters of disseminated intravascular coagulation (activated prothrombin time and fibrinogen/fibrin degradation products), although FR134043 seemed to have no effect on lipopolysaccharide-induced leukopenia. A relationship between elastase and the anticoagulant abnormality induced by sepsis has been reported (Anderssen et al., 1993; MacGregor et al., 1997). Our results also suggest that elastase plays an important role in anticoagulant abnormalities and that some elastase inhibitors will be effective in this state.

In the in vivo animal models, FR134043 in low doses protected against human neutrophil elastase-induced lung hemorrhage (i.t. and i.v. treatment), human neutrophil elastase-induced paw edema (s.c. treatment) and lipopolysaccharide-induced disseminated intravascular coagulation (continuous i.v. infusion) in rats. However, a high dose was needed to protect against porcine pancreatic elastase-induced emphysema (i.t. treatment) in hamsters.

In summary, FR134043 is a competitive inhibitor of human neutrophil elastase that inhibits both the amidolytic activity (synthetic substrate) and elastolytic activity (insoluble elastin) of elastase. Judged by the in vivo data for FR134043, systemic treatment with FR134043 has advantage over intratracheal treatment with FR134043. Our findings suggest that systemically administered FR134043 will

be a useful agent for studying the pathogenetic role of human neutrophil elastase in inflammatory diseases such as adult respiratory distress syndrome, pancreatitis, septic shock and rheumatoid arthritis.

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References

Adeyemi, E.O., Neumann, S., Chadwick, V.S., Hodgson, H.J.F., Pepys, M.B., 1985. Circulating human leukocyte elastase in patients with inflammatory bowel disease. Gut 26, 1306–1311.

Anderssen, T., Halvorsen, H., Bajaj, S.P., Osterud, B., 1993. Human leukocyte elastase and cathepsin G inactivate factor VII by limited proteolysis. Thromb. Haemost. 70, 414–417.

Baici, A., Pelloso, R., Horler, D., 1990. The kinetic mechanism of inhibition of human leukocyte elastase by MR 889: A new cyclic thiolic compound. Biochem. Pharmacol. 39, 919–924.

Bonney, R.J., Ashe, B., Maycoch, A., Dellea, P., Hand, K., Osinga, D., Fletcher, D., Mumford, R., Davies, P., Frankenfield, D., Nolan, T., Schaffer, L., Hagmann, W., Finke, P., Shah, S., Dorn, C., Doherty, J., 1989. Pharmacological profile of the substituted beta-lactam L-659, 286. A member of a new class human PMN elastase. J. Cell. Biochem. 39, 47–53.

Carp, H., Miller, F., Hoidal, J.R., Janoff, A., 1982. Potential mechanism of emphysema: α1-proteinase inhibitor recovered from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity. Proc. Natl. Acad. Sci. USA 79, 2041–2045.

Cox, D.W., Levison, H., 1988. Emphysema of early onset associated with a complete deficiency of alpha-1-antitrypsin (null homozygotes). Am. Rev. Respir. Dis. 137, 371–375.

Damas, J., Remacle-Volon, G., 1992. Influence of a long-acting bradykinin antagonist, Hoe140, on some acute inflammatory reactions in the rat. Eur. J. Pharmacol. 211, 81–86.

Finlay, G.A., O'Donnell, M.D., O'Connor, C.M., Hayes, J.P., FitzGerald, M.X., 1996. Elastin and collagen remodeling in emphysema: A scanning electron microscopy study. Am. J. Pathol. 149, 1405–1415.

Fletcher, D.S., Osinga, D.G., Hand, K.M., Dellea, P.S., Ashe, B.M., Manford, R.A., Davies, P., Hagmann, W., Finke, P.E., Doherty, J.B., Bonney, R.J., 1990. A comparison of α1-proteinase inhibitor methoxysuccinyl-Ala–Ala-Pro-Val-chloromethylketone and specific β-lactam inhibitors in an acute model of human polymorphonuclear leukocyte elastase-induced lung hemorrhage in the hamster. Am. Rev. Respir. Dis. 141, 672–677.

Fric, P., Kasatirek, E., Slaby, J., Marck, T., 1985. Effect of new oligopeptide inhibitors of elastase on acute experimental pancreatitis in the rat. Hepato-Gastroenterology 32, 206–209.

Fujie, K., Shinguh, Y., Hatanaka, H., Shigematsu, N., Murai, H., Fujita, T., Yamshita, M., Okamoto, M., Okuhara, M., 1993. FR901277, a novel inhibitor of human leukocyte elastase from Streptomyces resistomycificus. J. Antibiot. 46, 908–913.

Fujimoto, K., Ogawa, M., Saito, N., Kosaki, G., Minamimua, N., Yamamoto, T., 1980. A novel method of isolation and some characteristic properties of human pancreatic elastases. Biochim. Biophys. Acta 612, 262–267.

Fujimoto, K., Kubo, K., Saito, N., Shinozaki, S., Okada, K., Matsuzawa, Y., Kobayashi, T., Sekiguchi, K., 1995. Neutrophil elastase inhibitor reduces asthmatic responses in allergic sheep. Respir. Physiol. 100, 91–100.

Gadek, J.E., Pacht, E.R., 1990. The proteinase–antiproteinase balance within the human lung: Implications for the pathogenesis of emphysema. Lung, Suppl., pp. 552–564.

Green, B.G., Weston, H., Ashe, B.M., Doherty, J., Finke, P., Hagmann, W., Lark, M., Mao, J., Maycock, A., Moore, V., Mumford, R., Shah, S., Walakovits, L., Knight, W.B., 1991. PMN elastase: A comparison of the specificity of human isozymes and the enzyme from other species toward substrates and inhibition. Arch. Biochem. Biophys. 286, 284–292.

Groutas, W.C., 1987. Inhibition of leukocyte elastase and leukocyte cathepsin G for treatment of emphysema and related ailment. Med. Res. Rev. 7, 227–241.

Hansen, G., Schuster, A., Zubrod, C., Wahn, V., 1995. Alpha 1-proteinase inhibitor abrogates proteolyic and secretagogue activity of cystic fibrosis sputum. Respiration 62, 117–124.

Havemann, K., Gramse, M., 1984. Physiology and pathology of neutral proteinases of human granurocytes. Adv. Exp. Med. Biol. 164, 1–20.

Imura, Y., Terashita, Z., Nishikawa, K., 1986. Possible role of platelet activating factor (PAF) in disseminated intravascular coagulation (DIC), evidenced by use of a PAF antagonist, CV-3988. Life Sci. 39, 111–117.

Janoff, A., 1985. Elastases and emphysema. Am. Rev. Respir. Dis. 132, 417–433.

Kawabata, K., Suzuki, M., Sugitani, M., Imaki, K., Toda, M., Miyamoto, T., 1991. ONO-5046, a new inhibitor of human neutrophil elastase. Biochem. Biophys. Res. Commun. 177, 814–820.

Koo, K.W., Leith, D.E., Sherter, C.B., Snider, G.L., 1976. Respiratory mechanics in normal hamsters. J. Appl. Physiol. 40, 936–942.

Lai, L., Diamond, L., 1990. Inhibition of porcine pancreatic elastase-induced emphysema by eglin-c. Exp. Lung Res. 16, 547–557.

Larsson, G., 1978. Natural history and life expectancy in severe alpha-1 anti trypsin deficiency. Acta Med. Scand. 204, 345–352.

Lee, C., Ohno, H., Tanaka, M., Iwamura, H., Katsura, M., Terashima, H., Okegawa, T., Kawasaki, A., 1987. Difference of enzyme inhibitor spectrum of between gabexate mesilate and nafamostat mesilate as serine proteinase. Gendai Iryo 19, 1045–1053, in Japanese.

Lee, C.T., Fein, A.M., Lippmann, M., Holzman, H., Kimbel, P., Weinbaum, G., 1981. Elastolytic activity in pulmonary lavage fluid from patients with adult respiratory distress syndrome. N. Engl. J. Med. 304, 192–196.

Llewellyn-Jones, C.G., Harris, T.A., Stockley, R.A., 1996. Effect of fluticasone propionate on sputum of patients with chronic bronchitis and emphysema. Am. J. Respir. Crit. Care. Med. 153, 616–621.

Lucey, E.C., Stone, P.J., Christensen, T.G., Breuer, R., Snider, G.L., 1988. An 18-months study of the effects on hamster lung of intratracheally administered human neutrophil elastase. Exp. Lung. Res. 14, 671–686.

MacGregor, I.R., Perrie, A.M., Donnelly, S.C., Haslett, C., 1997. Modulation of human endothelial thrombomodulin by neutrophils and their release products. Am. J. Respir. Crit. Care. Med. 155, 47–52.

McGuire, W., Spragg, R.G., Cohen, A.B., Cochrane, C.G., 1982. Studies on the pathogenesis of adult respiratory distress syndrome. J. Clin. Invest. 69, 543–553.

Mohr, W., Wessinghage, D., 1978. The relationship between polymorphonucler granulocytes and cartilage destruction in rheumatoid arthritis. Z. Rheumatol. 37, 81–86.

Morris, S.M., Kagan, H.M., Stone, P.J., Snider, G.L., Albright, J.T., 1986. Ultrastructural changes in hamster lung 15 min to 3 hr after exposure to pancreatic elastase. Anat. Rec. 215, 523–535.

Nakajima, K., Powers, J.C., 1979. Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. J. Biol. Chem. 254, 4027–4032.

Nakao, A., Partis, R.A., Jung, G.P., Muller, R.A., 1987. SC-39026, a specific human neutrophil elastase inhibitor. Biochem. Biophys. Res. Commun. 147, 666–674.

Naughton, M.A., Sanger, F., 1961. Purification and specificity of pancreatic elastase. Biochem. J. 87, 156–163.

O'Connor, C.M., Gaffney, K., Keane, J., Southey, A., Byrne, N., O'Mahoney, S., Fitzgerald, C.M., 1993. Alpha 1-proteinase inhibitor, elastase activity, and lung disease severity in cystic fibrosis. Am. Rev. Respir. Dis. 148, 1665–1670.

Shinguh, Y., Imai, K., Yamazaki, A., Inamura, N., Shima, I., Wakabayashi, A., Higashi, Y., Ono, T., 1997. Biochemical and pharmacological characterization of FK706, a novel elastase inhibitor. Eur. J. Pharmacol. 337, 63–71.

Skiles, J.W., Fuchs, V., Chow, G., Skoog, M., 1984. Inhibition of human leukocyte elastase by N-substituted tripeptide trifluoromethyl keton. Res. Commun. Pathol. Pharmcol. 45, 339–345.

Sommerhoff, C.P., Krell, R.D., Williams, J.L., Gomes, B.C., Strimpler, A.M., Nadel, J.A., 1991. Inhibition of human neutrophil elastase by ICI 200, 355. Eur. J. Pharmacol. 193, 153–158.

Stone, P.H., Lucey, E.C., Snider, G.L., 1990. Induction and exacerbation of emphysema in hamsters with human neutrophil elastase inactivated reversibly by a peptide boronic acid. Am. Rev. Respir. Dis. 141, 47–52.

Uchida, M., Okajima, K., Murakami, K., Okabe, H., Takatsuki, K., 1995. Endotoxin-induced pulmonary vascular injury is mainly mediated by activated neutrophils in rats. Thromb. Res. 78, 117–125.

Williams, J.C., Stein, R.L., Strimpler, A.M., Reaves, B., Krell, R.D., 1991a. Biochemical and pharmacological characterization of ICI 186, 756; A novel, potent and selective inhibitor of human neutrophil elastase. Exp. Lung. Res. 17, 725–741.

Williams, J.C., Stein, R.L., Giles, R.A., Krell, K.O., 1991b. Biochemistry and pharmacology of ICI 200, 880, a synthetic peptide inhibitor. Ann. New York Acad. Sci. 624, 230–243.