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Pharmacological activities of TEI-8362, a novel inhibitor of human neutrophil elastase

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1 TEI-8362, 4-(N-(3-((3-carboxypropyl)amino)-8-methyl-1-oxo-4-azaisochromen-6-yl)carbamoyl)-4-((phenylmethoxy)carbonylamino)butanoic acid ($C_{26}H_{28}N_4O_9$) is a novel inhibitor of human neutrophil elastase (HNE). We evaluated its pharmacological profile *in vitro* and *in vivo*.

2 TEI-8362 demonstrated potent inhibition of HNE with a K_i value of 1.38×10^{-9} M. Its selectivity for HNE among a variety of proteases ranged from 163 fold to 68,000 fold in favour of HNE.

3 The pulmonary haemorrhage that occurred after i.t. instillation of HNE to hamsters was inhibited by either i.t., i.v., or inhalant administration of TEI-8362.

4 Intratracheal administration of lipopolysaccharide induced pulmonary neutrophilia. Twenty-four hours after lipopolysaccharide administration, the additional treatment with formyl-methionyl-leucyl-phenylalanine resulted in a specific neutrophil-dependent acute lung injury. In this model, lung injury was significantly attenuated by i.t., i.v., or inhalant administration of TEI-8362.

5 These pharmacological actions of TEI-8362 suggest that this drug has therapeutic value in the treatment of destructive lung diseases due to neutrophils.

Keywords: Human neutrophil elastase; synthetic elastase inhibitor; lung injury

Abbreviations: BAL, bronchoalveolar lavage; FMLP, formyl-methionyl-leucyl-phenylalanine; HNE, Human neutrophil elastase; LPS, lipopolysaccharide; TEI-8362, 4-(N-(3-((3-carboxypropyl)amino)-8-methyl-1-oxo-4-azaisochromen-6-yl)carbamoyl)-4-((phenylmethoxy)carbonylamino)butanoic acid

Introduction

Human neutrophil elastase (HNE) is a protease that is present in the azurophilic granules of neutrophils. This enzyme can digest many components of the extracellular matrix and it has been suggested to play roles in the metabolism of connective tissue and resolution of foreign substances.

The extracellular activity of HNE is regulated by natural protease inhibitors, such as α1-proteinase inhibitor, α2-macroglobulin, and secretory leukoprotease inhibitor. However, the excessive release of HNE accompanying neutrophil lysis or the action of HNE at sites of inflammation induces an imbalance between proteinase and antiproteinase. This results in tissue damage and functional disorders due to the enzyme's broad specificity and proteolytic intensity.

Indeed it has been reported that neutrophil elastase is significantly increased in sputum and bronchoalveolar lavage fluids of patients with various chronic respiratory diseases, such as chronic respiratory disease (Mikami, 1991), chronic obstructive pulmonary disease (Piccioni *et al*., 1992), bronchiectasis (Stockley, 1987), and cystic fibrosis (Goldstein & Döring, 1986) and that there is a positive correlation between the level of neutrophil elastase and the clinical symptoms.

TEI-8362, 4-(N-(3-((3-carboxypropyl)amino)-8-methyl-1-oxo-4-azaisochromen-6-yl)carbamoyl)-4-((phenylmethoxy)carbonylamino)butanoic acid, $C_{26}H_{28}N_4O_9$, developed by Teijin, is a novel synthetic inhibitor of neutrophil elastase. In this study, we evaluated its pharmacological profile *in vitro* and *in vivo*. We describe the effectiveness of TEI-8362 by intratracheal, inhalant, or intravenous administration in a specific neutrophil-mediated lung injury model induced by the combination of lipopolysaccharide (LPS) and formyl-methionyl-leucyl-phenylalanine (FMLP) as well as in an HNE-induced lung injury model.

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Methods

Materials

TEI-8362 was synthesized in our laboratories and its chemical structure is shown in Figure 1. The reagents used in this study and their sources were the following: human sputum elastase, which is equivalent to HNE (Twumasi & Liener, 1977), from Elastin Products Co., Inc. (Pacific, MO, U.S.A.); bovine pancreatic α-chymotrypsin, bovine pancreatic trypsin, human leukocyte cathepsin G, human plasma plasmin, human plasma thrombin, and FMLP, from Sigma Chemical Co. (St. Louis, MO, U.S.A.); *Pseudomonas aeruginosa* elastase, from Nagase Biomedicals (Kyoto, Japan); MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide, Suc-Ala-Ala-Pro-Phe-p-nitroanilide, N-α-Bz-Arg-p-nitroanilide HCl, and Ile-Phe-Lys-p-nitroanilide 2HCl, from Bachem Feinchemikalien AG (Budendorf, Switzerland); and LPS (*E. coli* 004), from Difco Laboratories (Detroit, MI, U.S.A.); FMLP and all substrates were dissolved in dimethylsulphoxide and diluted to the appropriate concentration in saline.

Determination of the kinetic inhibitory constant for HNE

Human sputum elastase was used as human neutrophil elastase without further purification (Fletcher *et al*., 1990; Shinguh *et*

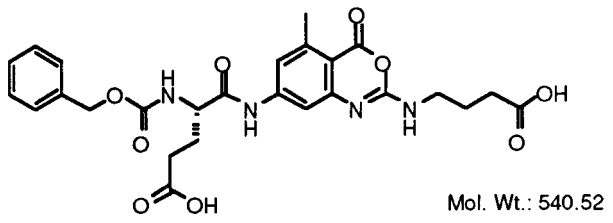

Figure 1 Chemical structure of TEI-8362, 4-(N-(3-((3-carboxypropyl)amino)-8-methyl-1-oxo-4-azaisochromen-6-yl)carbamoyl)-4-((phenylmethoxy)carbonylamino) butanoic acid ($C_{26}H_{28}N_4O_9$).

al., 1997). The assay mixture contained 50 μl of HNE (final concentration: 0.4 nM), 25 μl of substrate, i.e., MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide (final concentration: 0.1 mM), and 2.4 ml of 0.1 M HEPES (pH 7.5, with 1 M NaCl and 0.1% PEG 6000). The cuvette was placed in a thermostatically controlled spectrophotometer, and the reaction was started by addition of substrate. After a 0.8 min incubation, 25 μl of various concentrations (0, 1.6, 4.17, 6.25 and 12.5 nM) of inhibitor or dimethylsulphoxide was added to this reaction mixture; and the change in absorbance at 410 nm was continuously monitored at 37°C to calculate the change in the velocity of release of p-nitroaniline by HNE with and without inhibitor. Progress curves composed of more than 30 absorbance-time pairs were fitted to the equation of Neumann *et al*. (1991); Uejima *et al*. (1993).

Selectivity of TEI-8362 for serine proteinases

The reaction buffer used throughout this experiment was 0.1 M HEPES (pH 7.5), containing 0.5 M NaCl. The required inhibitor solution was preincubated for 10 min at 37°C with a variety of serine proteinases. The reaction was started by adding respective substrates and carried out for 20 min at 37°C. The light absorbance of the reaction mixture at 405 nm was measured. Residual enzyme activities were plotted for various concentrations of TEI-8362. IC_{50} was defined as the concentration of inhibitor giving rise to 50% inhibition of the hydrolysis.

Exogenous elastase-induced acute lung injury model in hamsters

Male Syrian Golden hamsters, 8–10 weeks old, were anaesthetized with halothane and HNE (50 μg per animal in saline) was administered intratracheally. The volume of HNE or vehicle was 100 μl. Three hours after HNE administration, bronchoalveolar lavage (BAL) was performed according to the method of Barry *et al*. (1990). Briefly, hamsters anaesthetized with urethane (1 g kg^{-1} intraperitoneally) were intubated endotracheally, and BAL was performed three times by the rewash lavage method (6 ml of PBS). As a marker of lung injury, the hemoglobin content of the BAL fluid was determined by the cyanmethemoglobin method (Matsubara & Shibata, 1969). Inhibition of HNE-induced lung hemorrhage was determined by dosing the hamster with TEI-8362 either intravenously 2 min before HNE treatment, intratracheally 1 h before HNE treatment, or with an aerosol 5 min after HNE treatment. Intravenous injection of TEI-8362 was carried out into the femoral vein under anaesthesia with halothane. In the inhalation study, hamsters were kept in an acrylic chamber (72 L; 35 × 45 × 42 cm) throughout TEI-8362 exposure. A solution containing 6 mg ml^{-1} TEI-8362 in saline was aerosolized by an ultrasonic nebulizer (NE-U12; Omron, Japan) for 15 min. This device produces an aerosol with a mass median diameter of 4.3 μm as determined by a laser deflection analyzer.

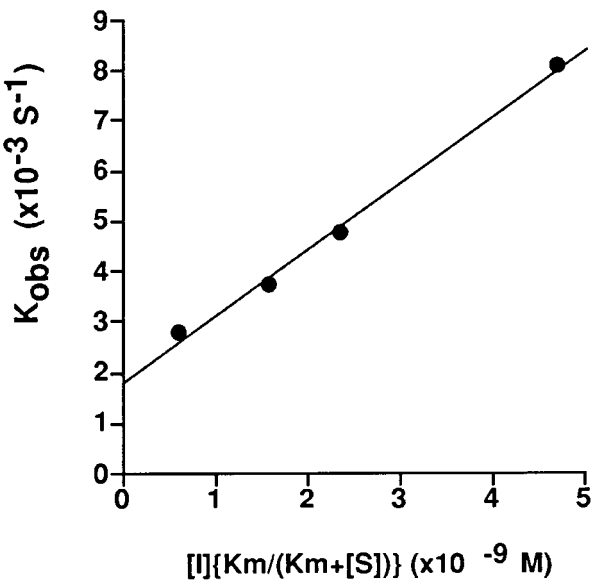

Figure 2 Kinetics of inhibition of HNE by TEI-8362. TEI-8362 is a slow-binding inhibitor of human neutrophil elastase. Progress curves composed of more than 30 absorbance-time pairs were fitted to the equation of Neumann *et al*. (1991), where K_{app}, [I], and [S] are the pseudo-first-order rate constant, inhibitor concentration and substrate concentration, respectively. K_{on} and K_{off} are derived from the gradient and the interception of plots on y axis, respectively.

Table 1 Inhibitory capacity and selectivity of TEI-8362 for serine proteinases

Enzyme (concentration; M)	IC_{50} (nM)
Human neutrophil elastase (4×10^{-10})	1.9
Human leukocyte cathepsin G (4×10^{-9})	6,100
Human plasma plasmin (9.6×10^{-11})	110,000
Human plasma thrombin (1×10^{-7})	130,000
Bovine pancreatic α-chymotrypsin (4×10^{-11})	1,900
Bovine pancreatic trypsin (1.7×10^{-8})	310

Chromogenic substrates (1×10^{-4} M): neutrophil elastase, MeO-Suc-Ala-Ala-Pro-Val-pNA; cathepsin G, Suc-Ala-Ala-Pro-Phe-pNA; plasmin, Ile-Phe-Lys-pNA. 2HCl; thrombin, Bz-Arg-pNA. HCl; α-chymotrypsin, Suc-Ala-Ala-Pro-Phe-pNA; trypsin, Bz-Arg-pNA. HCl.

Combination of LPS and FMLP-induced acute lung injury model in hamsters

The combination of LPS and FMLP is capable of making a specific neutrophil-mediated lung injury model, and this lung injury was shown to be mainly mediated by neutrophil proteases (Mitsuhashi *et al*., 1997). Hamsters anaesthetized with halothane were administered LPS, dissolved in saline, (0.32 mg ml^{-1} kg^{-1}) intratracheally. This treatment was followed 24 h later by intratracheal administration of FMLP, dissolved in saline with 0.04% dimethylsulphoxide (0.19 mg ml^{-1} kg^{-1}). Ninety minutes after FMLP administration, BAL was performed. Inhibition of lung haemorrhage was determined by dosing the hamster with TEI-8362 either intravenously 2 min before FMLP treatment, with an aerosol immediately after FMLP treatment, or intratracheally at the

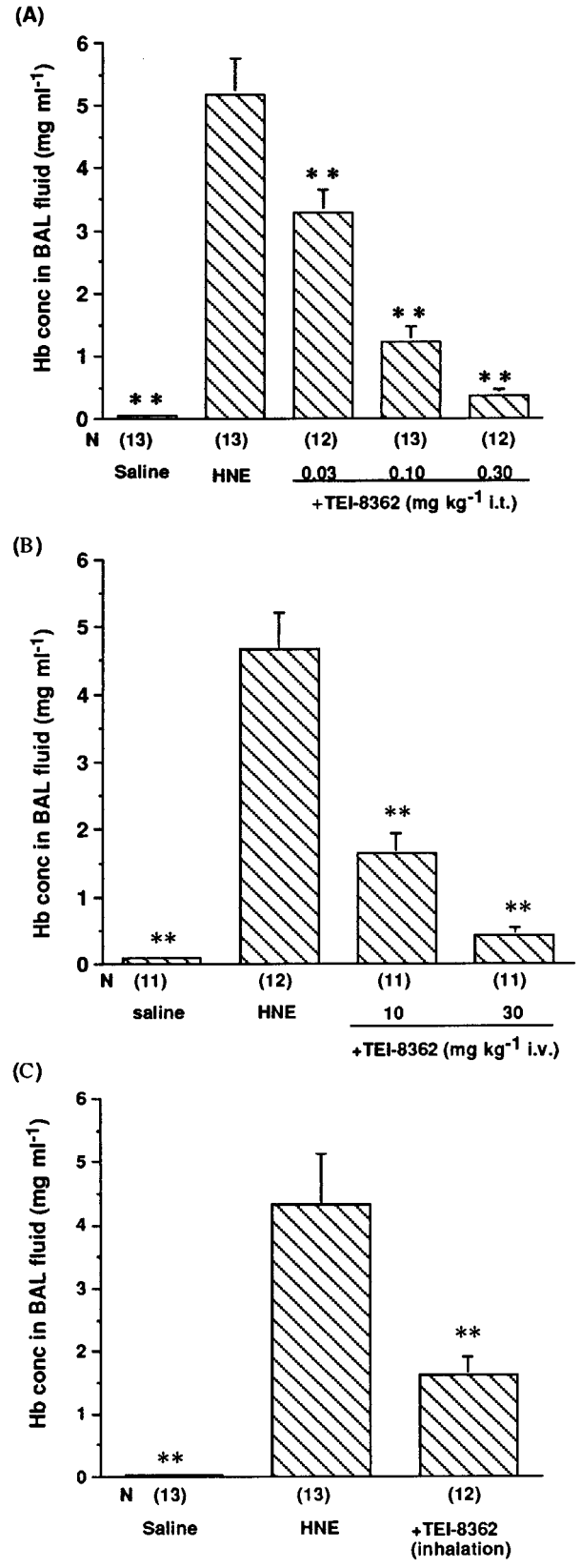

Figure 3 Effects of TEI-8362 on HNE-induced lung haemorrhage in hamsters. Each value is the means ± s.e.mean. TEI-8362 was administered intratracheally 1 h (A), intravenously 2 min (B) before HNE (50 μg per animal) instillation, or inhalation (C). In the inhalation study, TEI-8362 aerosol generated by nebulizing 6 mg ml^{-1} solution was administered inhalatively 5 min after HNE (50 μg per animal) instillation for 15 min. $^{**}P<0.01$, significantly different from HNE-treated group.

same time as FMLP treatment. In the inhalation study, a solution containing 5 mg ml^{-1} TEI-8362 in saline was aerosolized by an ultrasonic nebulizer for 15 min.

Assay for elastase activity

Elastase activity in BAL fluid was measured by use of a synthetic substrate of elastase, i.e., methoxysuccinyl-alanyl-alanyl-prolyl-valine-7-amido-4-methylcoumarin, according to Mario *et al.* (1979). Briefly, 75 μl of the supernatant of centrifuged BAL fluid was added to 1.4 ml of 0.05 M Tris buffer (pH 7.5) containing 0.5 M NaCl and 1 mM $CaCl_2$. This solution was incubated with 25 μl of 6 mM substrate at 37°C for 60 min. The fluorescence intensity was measured at excitation 370 nm and emission at 460 nm. A standard curve was constructed to convert relative fluorescence intensity into concentration of 7-amido-4-methylcoumarin released by enzymatic hydrolysis. By reference to a standard curve constructed with known concentrations of human sputum elastase, elastase activity in BAL fluid was presented as the concentration of human sputum elastase producing the same level of fluorescence.

Statistical analysis

The data are expressed as the means ± s.e.mean. Groups were compared by Student's standard two-tailed *t*-test and by Dunnett's mutliple comparison test when appropriate. Groups were considered different if the *P* value was less than 0.05.

Results

Inhibitory capacity and selectivity

As shown in Figure 2, the acylation constant K_{on} and the deacylation constant K_{off} of TEI-8362 were estimated to be 1.32×10^{6} M^{-1} S^{-1} and 1.82×10^{-3} S^{-1} respectively. The K_i value was estimated as the ratio of K_{on} to K_{off} (1.38×10^{-9} M). Selectivity of TEI-8362 for other serine proteinases is shown in Table 1. TEI-8362 showed selectivity for HNE versus chymotrypsin which was 1000 fold, and versus trypsin which was more than 100 fold in favour of HNE. Additionally, TEI-8362 did not inhibit *Pseudomonas aeruginosa* elastase (IC50 > 1 mM; data not shown).

Exogenous elastase-induced acute lung injury model in hamsters

First we evaluated the potency of TEI-8362 as an inhibitor of HNE-induced acute lung injury when administered intratracheally, intravenously, or as an aerosol. The assessment of HNE-induced acute lung injury was performed by measurement of the haemoglobin content in BAL fluid (Herbert *et al.*, 1992; Durham *et al.*, 1994; Shinguh *et al.*, 1997). Intratracheal administration of TEI-8362 (0.03, 0.10 and 0.30 mg kg^{-1}) 1 h before HNE administration inhibited acute lung haemorrhage in a dose-dependent manner (Figure 3a). Pulmonary haemorrhage was inhibited by 36.7, 76.9 and 93.4%, respectively, with an ED_{50} of 45 μg kg^{-1}. Intravenous injection of TEI-8362 (10 and 30 mg kg^{-1}) 2 min before HNE administration also inhibited acute lung haemorrhage in a dose-dependent manner (Figure 3b). In this case pulmonary haemorrhage was inhibited by 66.1 and 92.9%, respectively, with an ED_{50} of less than 10 mg kg^{-1}. Inhalant administration of TEI-8362 (6.0 mg ml^{-1} for 15 min from 5 min after HNE administra-

tion significantly inhibited acute lung haemorrhage (Figure 3c). The dose as an aerosol of TEI-8362 was estimated to be 91 μg kg^{-1} from measurement of the aerosol concentration with HPLC by the following calculated formulation:

Dose = (Aerosol concentration) × (Respiration volume) × (Inhalation time) × (Lung deposition ratio)/(Body weight)
Aerosol concentration = actual measurement by aersol sampling
Respiration volume = 4.19 × (Body weight)$^{0.66}$ (Hickson *et al*., 1992)
Lung deposition ratio = 0.13 (USEPA, 1982)

Combination of LPS and FMLP-induced acute lung injury model in hamsters

To evaluate drug effectiveness with a specific neutrophil-dependent model, we chose intratracheal administration of

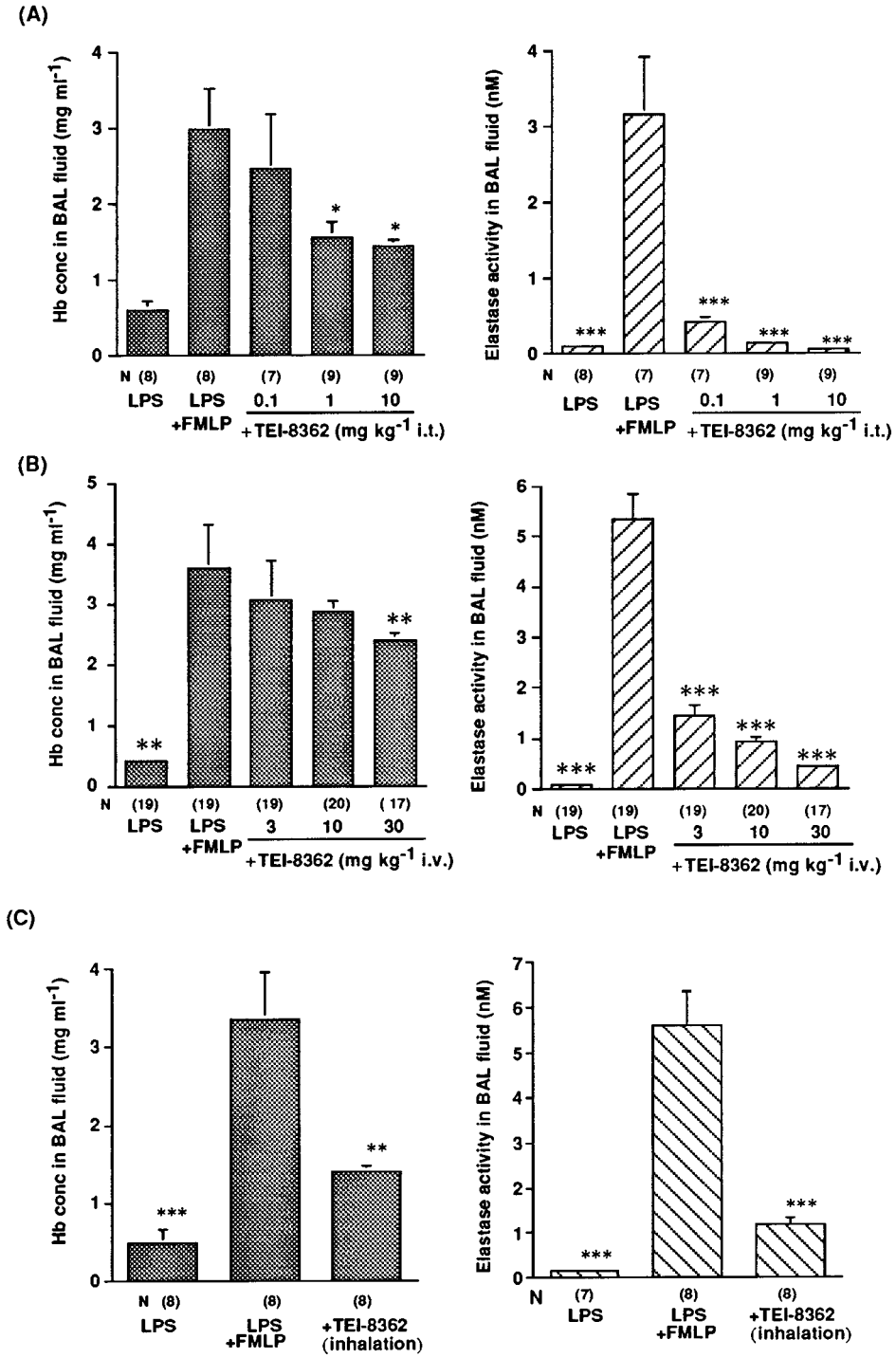

Figure 4 Effects of TEI-8362 on LPS and FMLP-induced acute lung injury in hamsters. Each value is the means ± s.e.mean. TEI-8362 was administered intratracheally simultaneously with FMLP administration (A), intravenously 2 min before FMLP treatment (B), or inhalation (C). In the inhalation study, TEI-8362 aerosol generated by nebulizing 5 mg ml^{-1} solution was administered by inhalation immediately after FMLP instillation for 15 min. *$P<0.05$, **$P<0.01$ and ***$P<0.001$, significantly different from LPS and FMLP-treated group.

FMLP, a neutrophil stimulator, 24 h after LPS administration. In this model, we investigated the effects of intratracheal, intravenous, or inhalant administration of TEI-8362. TEI-8362 administered intratracheally (0.1, 1.0 and 10.0 mg kg^{-1}) simultaneously with FMLP, inhibited acute lung haemorrhage and attenuated the increase in elastase-like activity in a dose-dependent manner (Figure 4a). Pulmonary haemorrhage was inhibited by 22.3, 59.4 and 64.5%, respectively, with an ED_{50} of 0.56 mg kg^{-1}. Intravenous injection of TEI-8362 (3, 10 and 30 mg kg^{-1}) 2 min before FMLP administration also inhibited acute lung haemorrhage dose-dependently (Figure 4b). Pulmonary haemorrhage was inhibited by 16.7, 23.0 and 38.1%, respectively, with an ED_{50} of more than 30 mg kg^{-1}. Inhalant administration of TEI-8362 (5.0 mg ml^{-1} for 15 min) from 5 min after FMLP administration significantly inhibited acute lung haemorrhage (Figure 4c). The dose as an aerosol of TEI-8362 was estimated to be 0.076 mg kg^{-1} from measurement of the aerosol concentration.

Discussion

It has been reported that neutrophil elastase is significantly increased in sputum and BAL fluids of patients with various respiratory diseases and thus it has been implicated in the tissue destruction associated with respiratory diseases. Recently, the action of neutrophil elastase has been reported to be not only destructive to tissue but also pathophysiological, causing induction of airway hyperresponsiveness (Suzuki *et al*., 1996), mucus secretion (Schuster *et al*., 1992; Tabachnik *et al*., 1992), genesis of secretory cell metaplasia (Lucey *et al*., 1990), smooth muscle cell proliferation (Thompson & Rabinovitch, 1996), modulation of leukocyte adhesion (Cai & Wright, 1996), induction of interleukin-8 gene expression (Nakamura *et al*., 1992), and impairment of phagocytic host defences against bacteria (Tosi *et al*., 1990). These findings suggest that inhibition of elastase would ameliorate such pathological and functional disorders.

In the presnet study TEI-8362 was shown to be a potent, selective inhibitor of HNE *in vitro*. The potency of TEI-8362 as an inhibitor of elastase activity *in vitro* is similar to or greater than that of other synthetic inhibitors such as ICI-200880 (Williams *et al*., 1991), ONO-5046 (Kawabata *et al*., 1991), MDL101,146 (Durham *et al*., 1994), and FK706 (Shinguh *et al*., 1997). Furthermore, significant inhibition was observed in *in vivo* models of HNE-induced acute lung injury when given either intratracheally, intravenously or as an aerosol (Figure 3). These results indicated that TEI-8326 will be a useful agent for studying the pathological role of human neutrophil elastase not only in respiratory diseases but also in other inflammatory disorders.

A number of elastase inhibitors have been evaluated for their activity to inhibit HNE-induced lung haemorrhage (Williams *et al*., 1991; Kawabata *et al*., 1991; Herbert *et al*., 1992; Durham *et al*., 1994; Shinguh *et al*., 1997). Attention has been focused on neutrophil elastase in lung injury because of the proteolytic intensity and broad specificity of this enzyme. However because it is known that neutrophils release a number of other toxic factors such as lipid metabolites, oxidants, and other proteases, the role of neutrophil elastase in the lung injury promoted by neutrophils remains unclear. Therefore, the HNE-induced injury model alone appears to be inappropriate for evaluation of an elastase inhibitor activity in lung damage. So, we undertook not only to evaluate TEI-8362 using the HNE-induced lung injury model but also to develop a specific neutrophil-mediated lung injury model for use in evaluating the potency of this drug. Intratracheal and intravenous administration of TEI-8362 inhibited acute lung haemorrhage in a dose-dependent manner accompanied by suppression of the increase in elastase-like activity in our neutrophil-mediated lung injury model (Figure 4a and b). These results indicate that the effect of TEI-8362 is mediated by elastase inhibition and suggest that TEI-8362, a neutrophil elastase inhibitor, might be useful in the treatment of destructive lung diseases due to neutrophils.

A greater dose of TEI-8362 was needed in the neutrophil-mediated lung injury model than in the HNE-induced model. Comparison of the effectiveness of TEI-8362 in the two models is difficult however, since the relative potency against hamster elastase is unknown. The degree of inhibition of haemorrhage by TEI-8362 was weak in comparison with its inhibition of elastase activity indicating the possible participation of toxic factors other than neutrophil elastase in neutrophil-mediated lung injury.

In the present study, we confirmed the effectiveness of TEI-8362 delivery by inhalant administration after instillation of the injury eliciting substance. Moreover, the calculated dose was small relative to that of intratracheal or systemic administration. This result suggests that inhalant administration is an efficient route for combating respiratory injury.

In conclusion, since TEI-8362 is a water-soluble inhibitor, it can be applied in either inhalant or intravenous formulation. In HNE-induced and neutrophil-mediated lung injury models, injury was significantly attenuated by intratracheal, intravenous or inhalant administration of TEI-8362. These pharmacological activities of TEI-8362 suggest that TEI-8362 has therapeutic value in the treatment of destructive lung diseases due to neutrophils.

References

BARRY, T.P., STEVEN, I., CASSANDRAY, M., LYNN, D.G. & ALLEN, B.C. (1990). A modified bronchoalveolar lavage procedure that allows measurement of lung epithelial lining fluid volume. *Am. Rev. Respir. Dis.,* **141,** 314–320.

CAI, T.-Q. & WRIGHT, S.D. (1996). Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, alpha M beta 2) and modulates polymorphonuclear leukocyte adhesion. *J. Exp. Med.,* **184,** 1213–1223.

DURHAM, S.L., HARE, C.M., ANGELASTRO, M.R., BURKHART, J.P., KOEHL, J.R., MARQUART, A.L., MEHDI, S., PEET, N.P. & JANUSZ, M.J. (1994). Pharmacology of N-[4-(4-morpholinyl-carbonyl) benzoyl]-L-valyl-N -[3,3,4,4,4-pentafluoro-1-(1-methylethyl)-2-oxobutyl]-L-prolinamide (MDL-101,146): A potent orally active inhibitor of human neutrophil elastase. *J. Pharmacol. Exp. Ther.,* **270,** 185–191.

FLETCHER, D.S., OSINGA, D.G., HAND, K.M., DELLEA, S.P., ASHE, B.M., MUMFORD, R.A., DAVIES, P., HAGMANN, W., FINKE, P.E., DOHERTY, J.B. & BONNEY, R.J. (1990). A comparison of α-1-proteinase inhibitor methoxysuccinyl-ala-ala-pro-val-chloromethylketone and specific β-lactam inhibitors in an acute model of human polymorphonuclear leukocyte elastase-induced lung haemorrhage in the hamster. *Am. Rev. Respir. Dis.,* **141,** 672–677.

GOLDSTEIN, W. & DÖRING, G. (1986). Lysosomal enzymes from polymorphonuclear leukocytes and proteinase inhibitors in cystic fibrosis. *Am. Rev. Respir. Dis.,* **134,** 49–56.

HERBERT, J.M., FREHEL, D., ROSSO, M.P., SEBAN, E., CASTET, C., PEPIN, O., MAFFRAND, J.P. & LE FUR, G. (1992). Biochemical and pharmacological activities of SR 26831, a potent and selective elastase inhibitor. *J. Pharmacol. Exp. Ther.,* **260,** 809–816.

HICKSON, M., ALEXANDER, D.J., SIBLEY, P.R., LIBRETTO, S.E., EZAKI, Y., MASUOKA, M. & TOMON, Y. (1992). Single inhalation toxicity study on fluticasone propionate in rats and dogs. *Jpn. Pharmacol. Ther.,* **20,** 1501–1508.

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.

LUCEY, E.C., STONE, P.J., CICCOLELLA, D.E., BREUER, R., CHRISTENSEN, T.G., THOMPSON, R.C. & SNIDER, G.L. (1990). Recombinant human secretory leukocyte-protease inhibitor: In vitro properties, and amelioration of human neutrophil elastase-induced emphysema and secretory cell metaplasia in the hamster. *J. Lab. Clin. Med.,* **115,** 224–232.

MARIO, J.C., KIICHIRO, N., MORRIS, Z. & JAMES, C.R. (1979). Sensitive substrates for human leukocyte and pancreatic elastase: A study of the merits of various chromophoric and fluorogenic leaving groups in assay for serine proteases. *Anal. Biochem.,* **99,** 53–64.

MATSUBARA, T. & SHIBATA, S. (1969). Evaluation of the internationally standardized method for haemoglobinometry. *Clin. Chim. Acta,* **23,** 427–430.

MIKAMI, M. (1991). Clinical and pathophysiological significance of neutrophil elastase in sputum and the effect of erythromycin in chronic respiratory disease. *Jpn. J. Thora. Dis.,* **29,** 72–83.

MITSUHASHI, H., ASANO, S., NONAKA, T., MASUDA, K. & KIYOKI, M. (1997). Potency of truncated secretory leukoprotease inhibitor assessed in acute lung injury models in hamsters. *J. Pharmacol. Exp. Ther.,* **282,** 1005–1010.

NAKAMURA, H., YOSHIMURA, K., MCELVANEY, N.G. & CRYSTAL, R.G. (1992). Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J. Clin. Invest.,* **89,** 1478–1484.

NEUMANN, U., STURZEBECHER, J., LEISTNER, S. & VIEWEG, H. (1991). Inhibition of chymotrypsin and pancreatic elastase by 4H-3, 1-benzoxazin-4-ones. *J. Enzyme Inhibition,* **4,** 227–232.

PICCIONI, P.D., KRAMPS, J.A., RUDOLPHUS, A., BULGHERONI, A. & LUISETTI, M. (1992). Proteinase/proteinase inhibitor imbalance in sputum sol phases from patients with chronic obstructive pulmonary disease. *Chest,* **102,** 1470–1476.

SCHUSTER, A., UEKI, I. & NADEL, J.A. (1992). Neutrophil elastase stimulates tracheal submucosal gland secretion that is inhibited by ICI 200, 355. *Am. J. Physiol.,* **262** (lung Cell. Mol. Physiol. 6), L86–L91.

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.

STOCKLEY, R.A. (1987). Bronchiectasis-New therapeutic approaches based on pathogenesis. *Clinics in Chest Medicine,* **8,** 481–493.

SUZUKI, T., WANG, W., LIN, J.-T., SHIRATO, K., MITSUHASHI, H. & INOUE, H. (1996). Aerosolized human neutrophil elastase induces airway constriction and hyperresponsiveness with protection by intravenous pretreatment with half-length secretory leukoprotease inhibitor. *Am. J. Respir. Crit. Care Med.,* **153,** 1405–1411.

TABACHNIK, E., SCHUSTER, A., GOLD, W.M. & NADEL, J.A. (1992). Role of neutrophil elastase in allergen-induced lysozyme secretion in the dog trachea. *J. Appl. Physiol.,* **73,** 695–700.

THOMPSON, K. & RABINOVITCH, M. (1996). Exogenous leukocyte and endogenous elastases can mediate mitogenic activity in pulmonary artery smooth muscle cells by release of extracellular matrix-bound basic fibroblast growth factor. *J. Cell. Physiol.,* **166,** 495–505.

TOSI, M., ZAKEM, H. & BERGER, M. (1990). Neutrophil elastase cleaves C3bi on opsonized pseudomonas as well as CR1 on neutrophils to create a functionally important opsonin receptor mismatch. *J. Clin. Invest.,* **86,** 300–308.

TWUMASI, D.Y. & LEINER, I.E. (1977). Proteases from purulent sputum. Purification and properties of the elastase and chymotrypsin-like enzymes. *J. Biol. Chem.,* **252,** 1917–1926.

UEJIMA, Y., KOKUBO, M., OSHIDA, J., KAWABATA, H., KATO, Y. & FIJII, K. (1993). 5-methyl-4H-3, 1-benzoxazin-4-one derivative: Specific inhibitor of human leukocyte elastase. *J. Pharmacol. Exp. Ther.,* **265,** 516–523.

U.S. ENVIRONMENTAL PROTECTION AGENCY. (1982). Air quality criteria for sulfur oxides and particulates. EPA-600/8-82-029c, II-28 - II-32.

WILLIAMS, J.C., STEIN, R.L., GILES, R.A. & KRELL, K.O. (1991). Biochemistry and pharmacology for ICI 200,880, a synthetic peptide inhibitor. *Ann. N.Y. Acad. Sci.,* **624,** 230–243.

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