FR901277†, A NOVEL INHIBITOR OF HUMAN LEUKOCYTE ELASTASE FROM *Streptomyces resistomycificus*

PRODUCING ORGANISM, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

KEIKO FUJIE, YASUHIKO SHINGUH, HIROSHI HATANAKA, NOBUHARU SHIGEMATSU, HIDETSUGU MURAI, TAKASHI FUJITA, MICHIO YAMASHITA, MASANORI OKAMOTO* and MASAKUNI OKUHARA

Exploratory Research Laboratories,
Fujisawa Pharmaceutical Co., Ltd.,
5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan

(Received for publication November 20, 1992)

A novel human leukocyte elastase inhibitor, FR901277 was discovered in the fermentation broth of *Streptomyces resistomycificus* No. 7622. FR901277 has a molecular weight of 961 and a molecular formula of $C_{47}H_{63}N_9O_{13}$. The mode of inhibition is competitive, with a *Ki* of 1.2×10^{-8} M. Oral administration of FR901277 at doses from 32 to 320 mg/kg significantly prevented human leukocyte elastase-induced foot edema in mice.

Human leukocyte elastase (HLE) is considered to be the most destructive enzyme present in the body. The enzyme hydrolyzes several connective tissue components such as elastin, proteoglycan and certain types of collagen. It is released from polymorphonuclear leukocytes by inflammatory stimuli and may play a role in destructive processes associated with chronic inflammatory diseases such as emphysema[2]. Therefore, inhibitors of HLE may have value in medicine.

In our search for microbially produced HLE inhibitors, we discovered FR901277. In this report, we address the identification and fermentation of the producing microorganism, as well as the isolation, physico-chemical and biological properties of the inhibitor.

Materials and Methods

Microorganism

Strain No. 7622 was isolated from a soil sample obtained at Hirono-cho, Fukushima Prefecture, Japan. A lyophilized sample of this strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession No. FERM BP-2306.

Taxonomic Characterization

The methods and media described by SHIRLING and GOTTLIEB[3], and WAKSMAN[4] were employed for the taxonomic study. The preparation of cells and the determination of the isomer of diaminopimelic acid were performed by the procedures of BECKER *et al.*[5].

Fermentation

A seed medium (200 ml) containing soluble starch 1%, sucrose 1%, glucose 1%, cotton seed meal 1%, Polypepton 0.5%, soybean meal 0.5% and $CaCO_3$ 0.1% prepared in tap-water was added to each of twelve 500-ml Erlenmyer flasks and was sterilized at 120°C for 30 minutes. A loopful of *Streptomyces*

† FR901277 is identical to WS7622A[1].

resistomycificus No. 7622 from a mature slant, grown on yeast extract-malt extract agar at 30°C for 2 weeks, was inoculated into each of the seed flasks. The inoculated flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 30°C for 3 days. The resultant seed culture was inoculated (1.5% rate) into 160 liters of sterile fermentation medium. The medium consisted of 4% Pine-Dex (starch hydrolysate, Matsuya Chemicals Co.), 1% gluten meal, 0.5% wheat germ, 0.5% potato protein and 0.2% $CaCO_3$, prepared in tap-water contained in a 200-liter stainless steel jar-fermentor. The pH of the medium was adjusted to 7.0 with NaOH prior to sterilization at 120°C for 30 minutes. The fermentation was carried out at 25°C for 5 days employing aeration at 160 liters/minute and stirring at 200 rpm.

The production of FR901277 in the fermentation was monitored by HPLC using a reverse phase column (YMC A-302 ODS 150×6 mm i.d., Yamamura Chemical Co.). The solvent system was methanol-acetonitrile-0.3% H_3PO_4 (120:15:180), and the detection wave length was 210 nm. The concentration of FR901277 in the fermentation broth at 120 hours after inoculation averaged 9.5 μg/ml.

Isolation

The fermentation broth (160 liters) was premixed with diatomaceous earth and filtered. Fifty liters of acetone were added to the mycelial cake with stirring. The mixture was allowed to stand at room temperature overnight, and was filtered. The extract was concentrated under reduced pressure to remove acetone. The filtrate (140 liters) and mycelial extract were combined, and were adsorbed onto polymeric adsorbent Diaion HP-20 (Mitsubishi Chemical Industrial Limited, 17 liters) and eluted with methanol. The eluate was concentrated to dryness. The dried material was chromatographed on silica gel (Kieselgel 60, Merck, 1.3 liters). The column was washed with ethyl acetate and the active substance was eluted with acetone-methanol (10:1). The active fraction was concentrated, and was rechromatographed on silica gel (0.5 liter) with stepwise elution using a solvent system composed of chloroform and methanol. The desired substance was eluted with 10% methanol in chloroform. The fraction was concentrated to dryness to give 3 g of yellow powder. Next, 50 mg of the yellow powder dissolved in 50 μl of methanol was subjected to HPLC, YMC-D-ODS-15B 30×250 mm (Yamamura Chemical Co.) with 60% aqueous methanol as the mobile phase with a flow rate of 20 ml/minute. The chromatogram was run 60 times, and the fractions containing FR901277 were combined and concentrated to dryness. The dried material was dissolved in a small amount of methanol and was allowed to stand overnight at room temperature to give 600 mg of FR901277 as colorless prisms.

Physico-chemical Properties

Uncorrected melting points were taken on a Yanagimoto micro melting point apparatus and IR spectra were measured on a Jasco A-102 IR spectrometer. Optical rotation was determined on a Jasco DIP-140 polarimeter using a 10 cm-microcell. ^1H NMR spectra (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured with a Bruker AM400wb spectrometer controlled with an ASPECT 3000 computer. The chemical shifts are reported in ppm relative to internal tetramethylsilane and coupling constants are expressed in Hz. Low-resolution FAB-MS spectra were obtained on a VG ZAB-SE mass spectrometer and amino acid analysis was performed on a HITACHI 835 amino acid analyzer.

Protease Inhibition Assay

The buffer used throughout the assay was 0.1 M HEPES, pH 7.5 containing 0.5 M NaCl. Twenty-five ml of 2 mM methoxysuccinyl-$(Ala)_2$-Pro-Val-*p*-nitroanilide (substrate) and 50 μl of inhibitor or vehicle were mixed in wells of 96 well-microtiter plate. The absorbance of the mixture at 415 nm was measured using a microplate reader (Corona Electric Co.). After the measurement, 25 μl of a solution of human sputum elastase (HSE, 6 μg/ml) was added and incubated for 30 minutes at room temperature. Then the absorbance at 415 nm was measured. Percent inhibition was determined using the formula: 100×(1-**A** inhibitor present/**A** inhibitor absent), where **A** is the absorbance after 30 minutes of incubation with enzyme minus the absorbance before the enzyme addition. The effects of inhibitors of other proteases were assayed similarly using 2 mM *N*-succinyl-$(Ala)_3$-*p*-nitroanilide as substrate for porcine pancreatic elastase (PPE, Type IV, 5 μg/ml final), 2 mM *N*-α-benzoyl-Arg-*p*-nitroanilide as substrate for bovine pancreatic trypsin (Type I, 16 μg/ml final) and 2 mM methoxysuccinyl-$(Ala)_2$-Pro-Met-*p*-nitroanilide as substrate for bovine pancreatic chymotrypsin (Type II, 1.5 μg/ml final). HSE was obtained from Elastin Products Company Inc., MO,

U.S.A., and was used as HLE[6,7]. All substrates, proteases and α-1-antitrypsin (α-1-AT, from human plasma) were purchased from the Sigma Chemical Co.

HLE-induced Foot Pad Edema in Mice

Six-week-old male C57BL mice were used for *in vivo* studies. HLE was dissolved in saline at a concentration of 0.2 mg/ml. Twenty-five μl of HLE solution were injected to the right foot pad of the mouse. The left foot pad was injected with 25 μl saline. FR901277 was suspended in 0.5% methylcellulose and given orally 15 minutes prior to HLE injection. Edema measurements were made using a dial thickness gauge. The thickness of the right and left foot pads was measured at 0.5, 1 and 2 hours after HLE injection. Foot edema was calculated by subtracing the thickness of the left foot pad (saline-treated) from right foot pad (HLE-treated). STUDENT's *t*-test was used to determine statistical significance.

Results

Taxonomic Characterization of the Producing Strain

The vegetative mycelium developed without fragmentation. The aerial mycelium branched monopodially and formed spiral chains and *Rectiflexibiles* chains of spores with more than 30 spores per chain. The spores had a smooth surface and were oval in shape with a size of $0.7 \sim 0.9 \times 0.8 \sim 1.1$ μm. Sclerotic granules, sporangia and zoospores were not observed. The aerial mycelium was bluish gray to brownish gray. Melanoid pigments were produced in tryptone-yeast extract broth and peptone-yeast extract-iron agar. Analysis of whole cell hydrolysates of strain No. 7622 showed the presence of LL-diaminopimelic acid.

The morphological and chemical characteristics of strain No. 7622 permitted a clear assignment of

Table 1. Characteristics of strain No. 7622 and IFO 12814.

Conditions	No. 7622	IFO 12814
Spore chain	Spiral, *Rectiflexibiles*	Spiral, *Rectiflexibiles*
Spore surface	Smooth	Smooth
Spore mass color	Gray	Gray
Mycelial pigments	Brown, violet brown, reddish brown	Brown, reddish brown
Soluble pigments	−	−
Melanoid pigment	+	+
Temperature range for growth (optimum)	12~34°C (27°C)	14~37°C (31°C)
Gelatin liquefaction	+	+
Milk coagulation	−	−
Milk peptonization	+	+
Starch hydrolysis	+	+
Decomposition of cellulose	−	−
Carbon utilization		
D-Glucose	+	+
L-Arabinose	+	+
D-Xylose	±	+
Inositol	+	+
Sucrose	+	+
D-Fructose	+	+
D-Mannitol	+	+
L-Rhamnose	+	+
Raffinose	−	+

+, Positive; ±, weakly positive; −, negative.

the organism to the genus *Streptomyces*[8,9]. Among the known species of *Streptomyces*, strain No. 7622 proved to resemble *Streptomyces resistomycificus*. The results of comparison of strain No. 7622 and *S. resistomycificus* IFO 12814 (type strain) are summarized in Table 1. Strain No. 7622 closely resembles IFO 12814 except for the temperature range of growth and utilization of raffinose. As these differences were considered to be insignificant, strain No. 7622 was identified as *Streptomyces resistomycificus*.

Physico-chemical Properties

The physico-chemical properties of FR901277

Table 2. Physico-chemical properties of FR901277.

Appearance	Colorless prisms
MP	250~252°C (dec.)
$[\alpha]_D^{23}$ (c=1.0, MeOH)	+36°
Elemental analysis	$C_{47}H_{63}N_9O_{13}\cdot 2H_2O$
Calcd:	C 56.56, H 6.77, N 12.63
Found:	C 56.65, H 6.62, N 12.27
FAB-MS (m/z)	984 $(M+Na)^+$
UV λ_{max}^{MeOH} nm (ε)	287 (3,600)
$\lambda_{max}^{0.1\,N\ HCl\text{-}MeOH}$ nm (ε)	286 (3,300)
$\lambda_{max}^{0.1\,N\ NaOH\text{-}MeOH}$ nm (ε)	298 (5,100)
IR (KBr) cm^{-1}	3400, 3300, 3060, 2980, 2940, 1735, 1710, 1690, 1670, 1660, 1640, 1540, 1520, 1470, 1380, 1330, 1300, 1260, 1220, 1200, 1160, 1130, 1090, 1000, 980, 940, 920

Fig. 1. ^1H NMR spectrum of FR901277 (400 MHz, CD_3OD).

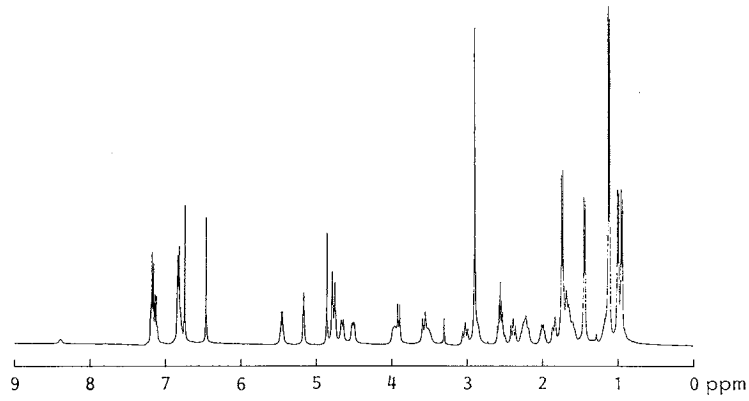
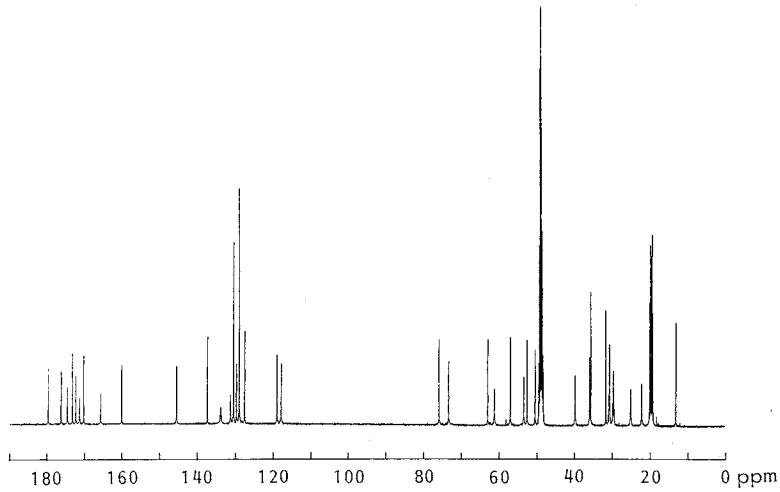


Fig. 2. ^{13}C NMR spectrum of FR901277 (100 MHz, CD_3OD).



are summarized in Table 2. FR901277 is soluble in methanol, slightly soluble in chloroform, ethyl acetate and acetone, and insoluble in water. Its color reactions were positive to ferric chloride and cerium sulfate, but negative to ninhydrin and Molisch reagents. The molecular formula was established as $C_{47}H_{63}N_9O_{13}$ by elemental analysis and FAB-MS. The ^1H NMR and ^{13}C NMR spectra of FR901277 are shown in Figs. 1 and 2. Amino acid analysis of a 6N HCl hydrolysate of FR901277 revealed the presence of Thr, Val, Orn, and NH_3 (1:1:1:1), plus three unknown amino acids. The details of the structure elucidation studies of FR901277 will be described elsewhere.

Biological Properties

Effects of FR901277 and α 1-AT on Various Proteinases

Inhibitory effects of FR901277 and α 1-AT against HLE, PPE, chymotrypsin and trypsin were determined. As shown in Table 3, FR901277 showed inhibition against HLE, PPE and chymotrypsin with IC_{50}s of 1.8×10^{-7}, 2.6×10^{-7} and 1.2×10^{-6} M, respectively. However, FR901277 was a very weak inhibitor of trypsin. α 1-AT, an endogenous inhibitor (MW=54 kDa) of serine protease, inhibited all enzymes tested with $IC_{50}s<10^{-6}$ M.

Table 3. Molar IC_{50} values of inhibitors for various proteinases.

Inhibitors	Proteinases			
	HLE	PPE	Chymotrypsin	Trypsin
FR901277	1.8×10^{-7}	2.6×10^{-7}	1.2×10^{-6}	3.3×10^{-4}
α 1-AT	4.4×10^{-8}	1.6×10^{-7}	5.8×10^{-8}	5.7×10^{-7}

Fig. 3. Lineweaver-Burk plot of inhibition of HLE by FR901277.

○ [I]=0, ● [I]=0.04, □ [I]=0.12 μM.

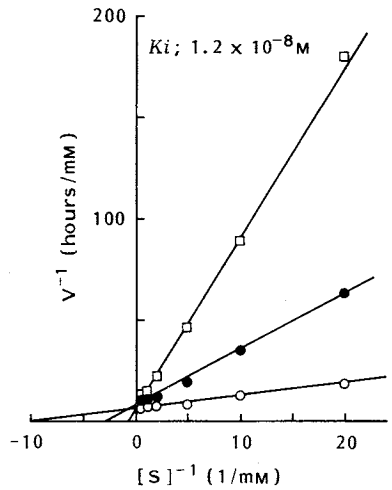
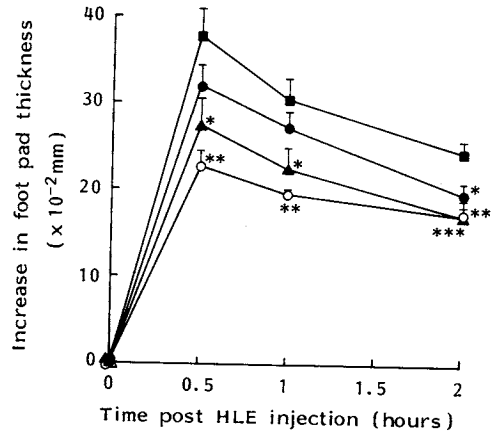


Fig. 4. Effect of FR901277 on HLE-induced paw edema in mice.

■ Control, ● 32, ▲ 100, ○ 320 mg/kg.



The paw edema was induced by 5 μg/site of HLE. FR901277 was administered orally 5 minutes prior to HLE injection. The increase in foot pad thickness was expressed as means±SEM, $\times10^{-2}$ mm, n=5. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Mode of Inhibition of FR901277

Lineweaver-Burk analysis[10] showed that the inhibition of HLE by FR901277 was competitive with substrate (Fig. 3). The *Ki* value was 1.2×10^{-8} M.

Effect of FR901277 on HLE-induced Paw Edema in Mice

To evaluate the *in vivo* activity of FR901277, we assesed the effect of the compound on HLE-induced paw edema in mice. Foot pad injection of HLE at 5 μg/site resulted in paw edema. The increase in the foot pad thicknesses were 37.8 ± 3.1, 30.4 ± 2.4 and $24.6 \pm 1.2 \times 10^{-2}$ mm (means $\pm$ SEM, $n=5$) at 0.5, 1 and 2 hours after HLE injection. As shown in Fig. 4, pretreatment with FR901277 at doses from 32 to 320 mg/kg p.o. significantly moderated edema.

Discussion

The development of potent *in vivo* active inhibitors of HLE such as FR901277 could lead to novel drugs for the treatment of pulmonary emphysema. FR901277 is an *in vivo* active inhibitor. The effect of FR901277 on a model of pulmonary emphysema is currently under study, and the results will be published later.

Acknowledgment

The authors thank Prof. N. OHSHIMA, Department of Biomedical Engineering, Institute of Basic Medical Sciences, University of Tsukuba, for critical reviewing this manuscript.

References

1) HATANAKA, H.; M. EZAKI, E. TSUJII, M. OKAMOTO, N. SHIGEMATSU, M. OKUHARA & S. TAKASE (Fujisawa Pharm.): New WS7622 compounds obtained from *Streptomyces resistomycificus* strain having human leukocyte elastase-inhibiting activity for treating degenerative diseases. Eur. Pat. 387 712, Sept. 19, 1990
2) POWERS, J. C.: Synthetic elastase inhibitors: prospects for use in the treatment of emphysema. Am. Rev. Respir. Dis. 127: 54~58, 1983
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Volume 2. Classification, Identification and Description of Genera and Species. The Williams and Wilkins Co., 1961
5) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
6) FLETCHER, D. S.; D. G. OSINGA, K. M. HAND, P. S. DELLA, B. M. ASHE, R. A. MUMFORD, P. DAVIES, W. HAGMANN, P. E. FINKE, J. B. DOHERTY & R. J. BONNEY: A comparison of α1-proteinase inhibitor methoxysuccinyl-Ala-Ala-Pro-Val-chromomethylketone 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, 1990
7) SKILES, J. W.; V. FUCHS, G. CHOW & M. SKOOG: Inhibition of human leukocyte elastase by *N*-substituted tripeptide trifluoromethyl ketone. Res. Commun. Chem. Pathol. Pharmacol. 45: 339~345, 1984
8) BUCHANAN, R. E. & N. E. GIBBONS (*Ed.*): BERGEY's Manual of Determinative Bacteriology. 8th Ed. The Williams and Wilkins Co., 1974
9) WILLIAMS, S. T. (*Ed.*): BERGEY's Manual of Systematic Bacteriology. Volume 4. Williams and Wilkins, 1989
10) LINEWEAVER, H. & D. BURK: The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56: 658~666, 1934