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Pharmacological Profile of the Substituted Beta-Lactam L-659,286: A Member of a New Class of Human PMN Elastase Inhibitors

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Human polymorphonuclear leukocyte elastase (PMN elastase) is inhibited by L-659,286 (7α-methoxy-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triaz-in-3-yl)thio]methyl]-5-thia-1-aza-6R-bicyclo[4.2.O]oct-2-ene-2-pyrrolidine carboxamide-5,-dioxide) with a K_i of 0.4 μM. This inhibition is time-dependent, rapid, and only slowly reversible, with a t1/2 of > 3 days at 25°C. L-659,286 is also highly selective for PMN elastase, as it does not inhibit thrombin, trypsin, papain, plasmin, chymotrypsin, or cathepsin G. L-659,286 administered intratracheally inhibits lung damage caused by administration via the same route of human PMN elastase into hamsters. In marmosets, L-659,286 is cleared from blood very rapidly after an intravenous injection but is recovered in bronchoalveolar lavage fluid for several hours after intratracheal administration.

Key words: elastase inhibitors, β-lactams, lung damage

Human polymorphonuclear leukocyte elastase (PMN elastase), a serine proteinase unique to this cell, hydrolyzes several connective tissue components such as elastin, proteoglycan, and certain types of collagen. This enzyme can be released from PMN by inflammatory stimuli and may play a role in the destructive processes associated with chronic inflammatory diseases such as emphysema [1]. In this disease the natural plasma inhibitor of PMN elastase, α1 proteinase inhibitor (α_1PI), is thought to have a diminished capacity to protect host connective tissues from degradation by the enzyme. In addition, individuals deficient in the natural inhibitor, having the so-called Pi_{ZZ} phenotype of α_1PI, are particularly susceptible to development of emphysema at an early age [2]. Therefore low molecular weight selective inhibitors

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of PMN elastase that can be delivered to the site of unregulated PMN elastase activity are needed. We have identified a class of cephalosporin-based compounds that bear a methoxy group in the 7 position and an ester or amide in the 4 position of the cephalosporin nucleus as potent inhibitors of human PMN elastase [3]. This manuscript describes the activity of one such compound, 7α-methoxy-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triazin-3-yl)thio]methyl]-5-thia-1-aza-6R-bicyclo[4.2.0]oct-2-ene-2-pyrrolidine carboxamide-5,-dioxide (L-659,286).

MATERIALS AND METHODS

Biochemistry

Human PMN elastase (Elastin Products, St. Louis, MO) was assayed spectrophotometrically at 25°C by continuous monitoring of the release of p-nitroaniline from Suc-Ala-Ala-Pro-Ala-p-nitroanilide at 410 nm. Incubation mixtures contained inhibitor, 0.2 mM substrate (both added in dimethyl sulfoxide [DMSO], 10% final concentration), and enzyme (added last) in 0.05 M TES, pH 7.5.

Elastin solubilization was determined as follows: incubation mixtures of 0.2 ml contained 125 μg ^{3}H-elastin (N.E.N., Boston, MA) and 0.4 μg human PMN elastase in Tris buffer, pH 7.5, containing 1% ovalbumin and 5% DMSO. The mixtures were incubated 1 h at 37°C with shaking and then centrifuged to remove the remaining insoluble substrate. The supernatant fluid was assayed for tritium to determine the extent of elastin solubilization as follows: 75 μl of supernatant fluid was added to 2.5 ml of Aquasol and counted in a Packard Tri-carb liquid scintillation counter. Experiments were performed in quadruplicate by premixing human PMN elastase with L-659,286 (added in 10 μl DMSO to achieve final concentrations of 0–60 μM) and then adding elastin. Percent of inhibition was calculated by comparing the degree of solubilization with that observed in incubation mixtures that lacked L-659,286.

Specificity studies were performed as follows: human cathepsin G [4] and human pancreatic chymotrypsin (a gift from C. Largman, University of California) were assayed by monitoring the increase in absorbance at 348 nm resulting from the release of *p*-nitrophenol from *t*-Boc-Tyr-ONp in pH 6.5 PIPES buffer containing 10% DMSO. Human thrombin and plasmin (Boehringer Mannheim) were assayed in the same manner using 0.2 mM Tosyl-Gly-Pro-Arg-*p*-nitroanilide as substrate. Papain (Boehringer Mannheim) was assayed as follows: enzyme was diluted 1:10 into a solution of benzoyl-Arg-*p*-nitroanilide in 50 mM TES, pH 7.5, 0.001 M EDTA, 0.005 M cysteine and 10% DMSO, and the increase in absorbance at 410 nm was monitored. Human pancreatic elastase and trypsin was purified and assayed as described by Largman et al. [5]. Acetylcholinesterase was assayed by monitoring the decrease in absorbance at 420 nm caused by the hydrolysis of acetylcholine chloride (6 mg/ml) in the presence of m-nitrophenol (0.45 mg/ml) in 0.04 M phosphate, pH 7.8. The percent of inhibition in all cases is calculated by comparison with control activities.

Cell Biological Studies

Isolation of PMN. Fresh human venous blood was centrifuged at 2,400 rpm for 5 minutes. The plasma was aspirated and the cells reconstituted to original volume with normal saline. Dextran (Sigma), (3%) was added at a ratio of 3 ml for every 10

ml of cells and incubated at room temperature for 30 min. The top layer containing the PMN was collected. One ml of Ficol-Hypaque (1077) was added for every 4 ml of cells to the bottom of the tubes to set up a cushion gradient. The mixture was centrifuged for 22 min at 1,000 rpm. The supernatant fluid was discarded. The pellet was dislodged, and an excess of red blood cell lysing solution consisting of 150 mM NH_4Cl, 0.11 mM EDTA, 9.99 mM KCO_3H was added, and the sample was agitated for 3 min. This preparation was centrifuged at 2,000 rpm for 5 min, and the supernatant fluid was decanted.

Incubation of PMN. The pelleted PMN were suspended (5×10^6/ml) in Hank's balanced salt solution (HBSS) and 0.1 M phosphate buffer, pH 7.2, containing 5 mM EDTA and divided into 1-ml aliquots. L-659,286 was added to selected aliquots, and all samples were incubated for 15 min at 37°C with shaking. Cells were washed three times by centrifugation with 2 ml of suspension buffer and resuspended in 0.5 ml of buffer without EDTA. A 2-μl aliquot of cytochalasin B (2.5 μg/ml in DMSO) was added to each and the mixtures were incubated for 5 min at 37°C. A 5-μl aliquot of 10^{-5} M formyl-methionyl-leucyl-phenylalanine (fMLP) was then added, and the incubations were continued for 15 min. Samples were centrifuged at 400*g* for 5 min and supernatants were assayed for elastase activity by the spectrophometric assay.

In Vivo Studies

Human PMN elastase-mediated damage in hamster lung. Human PMN elastase, 50 units, was injected into the trachea of anesthetized animals, causing hemorrhage into the air spaces of the lung. L-659,286 was injected intratracheally 30 min prior to the enzyme, and the decrease in hemorrhage into the lung was determined as follows: lung was lavaged with 2.5 ml of saline, and the amount of hemoglobin in the fluid was measured. Percent of inhibition was calculated by dividing the amount of hemorrhage occurring in the lungs of L-659,286-treated animals by the hemorrhage occurring in enzyme only-treated animals. The assay is similar to that described by Hassall et al. [6] and will be published in detail elsewhere.

Pharmacokinetics in Marmosets

Intratracheal administration. L-659,286 (400 μg) was administered intratracheally to *S. labiatus* marmosets. At various times, the lungs were lavaged with 5 ml saline. The cells were removed by centrifugation, and 50 μl of the supernatant were applied to a Supelcosil C-18 column (0.5 × 25 cm) equilibrated with H_2O:acetonitrile:trifluoroacetic acid, 80:20:0.2 v/v/v. The amount of L-659,286 present in each sample was calculated from a standard curve.

Intravenous (IV) administration. Adult marmosets were anesthetized with an intramuscular injection of acepromazine and ketamine. Lactated Ringer's solution was administered through a right femoral vein catheter at a rate of 30 ml/kg/h. L-659,286 (10 mg/kg) was injected IV, and blood samples (200 μl) were removed from the left femoral catheter at various time intervals. Duplicate 100-μl aliquots of blood were treated with 5.0 ml of acetonitrile-0.2 trifluroacetic acid (ACN-0.2% TFA). The samples were vortexed and centrifuged at 2,000 rpm for 10 min and the ACN layer was evaporated to dryness at 50°C under N_2. The samples were resuspended in

HPLC buffer and microfuged at 13,000 rpm for 3 min. A 50-μl aliquot of each sample was chromatographed on a Supelcosil C-18 column (0.4 × 25 cm) previously equilibrated with H_2O:ACN:TFA 87:13:0.2 v/v/v.

RESULTS

Biochemical Studies

L-659,286 (Fig. 1) inhibits human PMN elastase in a time-dependent nature. The progress curve for enzyme activity in the presence of L-659,286 is shown in Figure 2. Experiments at various concentrations of inhibitor demonstrated that the rate of inactivation was saturable, and from that curve the individual kinetic constants ($K_i = 0.4 \times 10^{-6}$ M and $k_{inact} = 0.0049\ s^{-1}$) were determined; ($k_{inact}/K_i = 12{,}800\ M^{-1}\ s^{-1}$). K_i is the concentration of inhibitor giving a half-maximum rate of

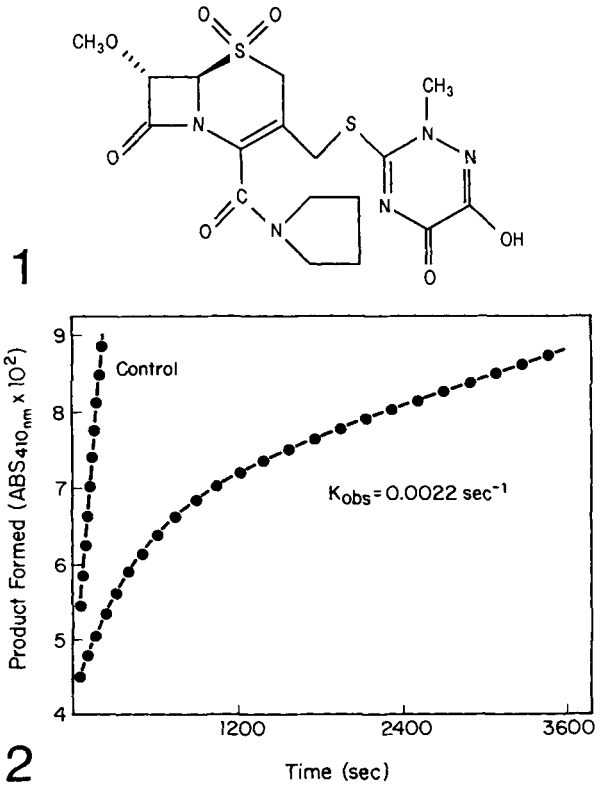

Fig. 1. Structure of L-659,286: 7α-methoxy-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triazin-3-yl)thio]methyl]-5-thia-1-aza-6R-bicyclo[4.2.0]oct-2-ene-2-pyrrolidine carboxamide-5,-dioxide.

Fig. 2. Kinetics of inhibition of elastase by L-659,286. Human PMN elastase was assayed spectrophotometrically with and without compound at 25°C by continuous monitoring of the release of nitroaniline from Suc-Ala-Ala-Pro-Ala-p-nitroanilide at 410 nm. The incubation mixture contained L-659,286 0.2 mM substrate, 10% DMSO, and 3.3×10^{-8} M enzyme (added last) in 0.05 M TES buffer, pH 7.5, 0.15 M NaCl. Absorbance was measured every 30 s, and the data were fit by nonlinear regression. Values of the initial velocity (V_0), final velocity (V_s), and first-order rate constant (k_{obs}) were obtained from the curve. The circles represent experimental points, and the line is theoretical.

inactivation, and k_{inact} is the first order rate constant for inactivation of enzyme saturated with inhibitor.

Upon standing, human PMN elastase inactivated with L-659,286 regained activity slowly with a $t^{1/2}$ of approximately 72 h at 25°C.

L-659,286 also inhibits the hydrolysis of elastin, a large molecular weight substrate of elastase. The IC_{50} (concentration giving 50% inhibition) for L-659,286 to inhibit elastinolysis by human PMN elastase is between 1 and 2 μg/ml (Fig. 3).

L-659,286 is quite specific for human PMN elastase, since at a concentration of 42 μM it did not have any effect on thrombin, plasmin, cathepsin G, trypsin, chymotrypsin or papain (Table I).

Cell Biological Studies

PMN elastase may serve an important role in the intracellular digestion and degradation of infectious agents after their phagocytosis by the cells. Thus it was important to determine if L-659,286 inhibits intracellular elastase. As shown in Table

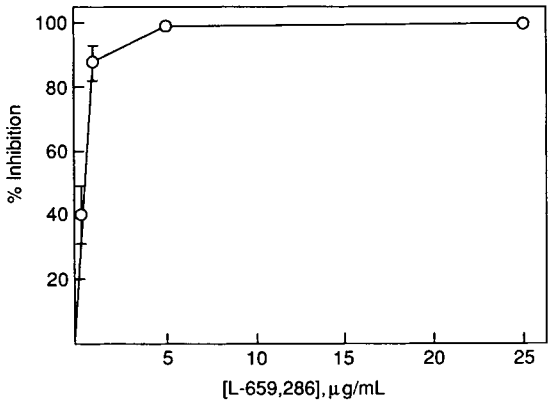

Fig. 3. Inhibition of elastin solubilization by L-659,286. Incubation mixtures contained in 0.2 ml total volume 125 μg ^{3}H-elastin and 0.4 μg human PMN elastase in Tris buffer, pH 7.5, containing 1% ovalbumin and 5% DMSO. Inhibitor was added in 10 μl DMSO. The mixtures were incubated for 1 hr at 37°C with shaking and then centrifuged to remove the remaining insoluble substrate. The supernatant fluid was counted for tritium to determine the extent of elastin solubilization. Experiments were performed in quadruplicate by premixing human PMN elastase and L-659,286.

TABLE I. Selectivity of L-659,286 as an Inhibitor of PMN Elastase*

Enzyme	% Inhibition
Thrombin (human)	10
Plasmin (human)	0
Cathepsin G (human)	3
Chymotrypsin (human)	44
Papain	6
Acetylcholine esterase	0
Pancreatic elastase (human)	6

*L-659,286 was added at 42 μM. PMN elastase is inhibited 100% by 0.1 μM L-659,286.

II, the supernatant fluid from cells pretreated with L-659,286 and then washed free of the drug before stimulation of the cells contains as much active PMN elastase as cells not exposed to the inhibitor.

In Vivo Studies

L-659,286, 100 μg, administered intratracheally to hamsters 30 min prior to intratracheal injection of 50 μg of human PMN elastase, prevented the resulting hemorrhage into the air spaces of the lung (Table III). As a negative control, the β-lactam antibiotic Cephalothin, which has a structure similar to L-659,286, but which lacks elastase inhibitory activity, was not active in this model at 400 μg/animal.

The activity of L-659,286 via the intratracheal route may be attributed to its retention in the lung, since the compound can be detected in bronchoalveolar lavage fluid of marmosets for several hours after an intratracheal dose of 400 μg (Fig. 4). In contrast, L-659,286 is cleared rapidly from the circulation of marmosets following an intravenous injection of 10 mg/kg (Fig. 4).

CONCLUSIONS

These studies document that a modified β-lactam structure (L-659,286) is a potent and selective inhibitor of human PMN elastase in vitro and in vivo. The k_{inact}/K_i for L-659,286 of 12,800 M^{-1} s^{-1} compares favorably with 3,4-dichloroisocumarin (8,920) [7] and to chloromethylketone (3,500) [8]. L-659,286 added to incu-

TABLE II. L-659,286 Does Not Inhibit Intracellular Elastase of Human PMN

	Conditions[a]			Results
Tube	Compound[b]	Wash	Cyto B + fMLP	(units of PMN elastase)
1	−	+	−	2
2	−	+	+	116
3	+	+	+	108
4	+	−	+	2
5	−	−	+	102

[a]Cells were preincubated with inhibitor for 15 min at 37°C, washed three times, resuspended, and treated with cytochalasin B (cyto B) plus fMLP for 15 min at 37°C. Supernatants were assayed for elastase as described [3].
[b]L-659, 286 at 10 μg/ml (21 μm).

TABLE III. Effect of L-659,286 and Cephalothin on Human PMN Elastase-Induced Lung Damage in Hamsters*

Compound μg	% Inhibition
L-659,286	
100	91 ± 7
10	18 ± 18
Cephalothin	
400	21 ± 32

*Compounds administered intratracheally 30 min prior to enzyme. Results are average ± SD, n = 4.

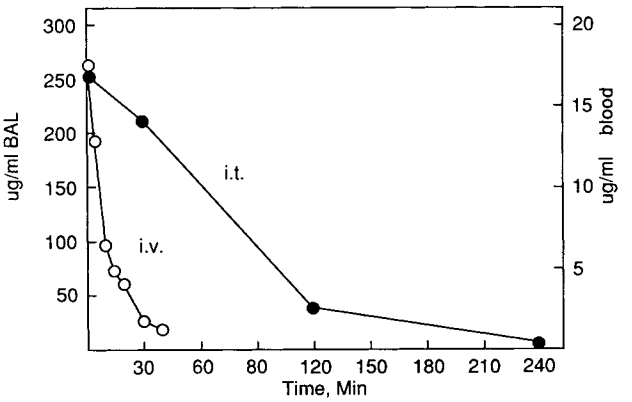

Fig. 4. Pharmacokinetics of L-659,286. Details are presented in Materials and Methods. Each point represents the average of two analyses from two animals. i.t., intratracheal; i.v., intravenous.

bated human PMN can be completely washed from the cells, allowing intracellular elastase to be released as active enzyme, showing that this compound does not inhibit intracellular elastase. When administered locally, the compound prevents lung damage in hamsters treated intratracheally with human PMN elastase. The compound persists in the lung for several hours after direct instillation but is cleared rapidly from the circulation after intravenous administration. These properties suggest that compounds like L-659,286 are suitable for aerosol administration to increase the elastase-inhibitory capacity in the terminal airways of the lung, which are susceptible to damage by the enzyme and subsequent development of emphysema.

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