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THE KINETIC MECHANISM OF INHIBITION OF HUMAN LEUKOCYTE ELASTASE BY MR889, A NEW CYCLIC THIOLIC COMPOUND

ANTONIO BAICI,*† RENZO PELLOSO‡ and DOROTHY HÖRLER*

*Department of Rheumatology, University Hospital, CH-8091 Zurich, Switzerland; and
‡Medea Research Ltd, Via Pisacane 34/a, I-20129 Milan, Italy

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Abstract—The cyclic thiolic compound 2-[3-thiophencarboxythio]-*N*-[dihydro-2(3H)-thiophenone-3-il]-propionamide (MR889) was investigated as inhibitor of endopeptidases. The activity of bovine pancreatic α-chymotrypsin, human leukocyte cathepsin G and rabbit liver cathepsin B was not affected by MR889, whereas porcine pancreatic elastase and human leukocyte elastase were inhibited. The kinetic mechanism of inhibition of human leukocyte elastase was of the reversible, slow-binding, fully competitive type. The rate constants for complex formation between MR889 and leukocyte elastase, determined by pre-steady-state kinetic analysis in the presence of a tetrapeptide substrate at 37° and pH 7.40, were k_{on} = 2363 ± 15 M^{-1} sec^{-1}, k_{off} = 3.01 ± 0.34 × 10^{-3} sec^{-1}. The inhibition equilibrium constant was $K_i = k_{off}/k_{on}$ = 1.27 ± 0.15 μM. K_i, calculated from steady-state kinetic experiments, was 1.38 μM. MR889 also inhibited the elastolytic activity of leukocyte elastase, as determined with insoluble elastin as the substrate.

Human leukocyte elastase is possibly one of the most destructive enzymes in the body, having the ability to degrade many components of the extracellular matrix such as insoluble collagens type I and II [1], type III collagen [2], type IV collagen [3, 4], proteoglycans [1, 5] and elastin [6, 7]. Other natural substrates degraded by leukocyte elastase are the four human immunoglobulin G subclasses [8], immunoglobulin M [9] and the cell adhesion molecule fibronectin [10]. For these reasons leukocyte elastase has been associated with pathologic states characterized by an abnormal degradation of connective tissue, in particular with pulmonary emphysema [11, 12], rheumatoid arthritis [13], clotting disorders and other inflammatory processes [14]. An attractive approach for the treatment of emphysema is the use of low molecular mass synthetic inhibitors of leukocyte elastase [15–17].

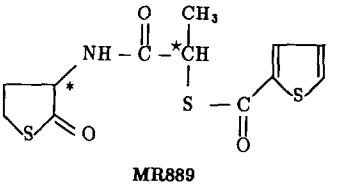
The present paper continues a series of studies on human leukocyte elastase inhibitors [18–24] and presents a kinetic characterization of the interaction between leukocyte elastase and MR889, a thiolactic acid derivative with mucoregulatory and broncho-secretory properties [25].

MATERIALS AND METHODS

Enzyme substrates. All peptide substrates and the elastase inhibitor MeO—Suc—Ala—Ala—Val—CH_2Cl§ were from Bachem Ltd (Bubendorf, Switzerland).

Enzymes. Leukocyte elastase (EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) were purified from human blood polymorphonuclear leukocytes as detailed elsewhere [26]. The concentration of the leukocyte elastase active sites was determined by titration with an irreversible inhibitor: an enzyme aliquot was incubated with increasing known amounts of MeO—Suc—Ala—Ala—Pro—Val—CH_2Cl and the residual activity was measured with Suc—Ala—Ala—Val—*p*-nitroanilide as substrate. Cathepsin B (EC 3.4.22.1) was purified from rabbit liver [27]. Crystalline α-chymotrypsin (EC 3.4.21.1) from bovine pancreas was a product of the Sigma Chemical Co. (St Louis, MO).

Inhibitor. 2-[3-Thiophencarboxythio]-*N*-[dihydro-2(3H)-thiophenone-3-il]-propionamide, or simply MR889 (see structural formula below), was synthesized as described [25]. Analysis (calculated/found %): C (45.71/45.67), H (4.13/4.17), N (4.44/4.49). The melting point was 129–131°. The molecule of MR889 has two centres of asymmetry: carbon 1′ of the thiolactone ring and carbon 2 of propionamide (marked in the structural formula with * and ★, respectively). In a couple of stereoisomers taken at random either an enantiomerism or a diastereoisomerism relationship can exist. The product used in this study consisted of a 1:1 mixture of diastereoisomers, with each of them being a 1:1 mixture of enantiomers.



MR889

† Author to whom correspondence should be addressed.

§ Abbreviations used: Names of amino acids and substituents are abbreviated in accordance with IUPAC-IUB recommendations [*Biochem J* **219**: 345–373, 1984]. Additional abbreviations: —CH_2Cl, chloromethane; —NMec, 7-(4-methyl)coumarylamide.

Enzyme assays. Kinetic experiments with leukocyte elastase were carried out using the fluorogenic tetrapeptide MeO—Suc—Ala—Ala—Pro—Val—NMec as substrate [28]. The buffer system was 57 mM Na^+/K^+ phosphate, pH 7.40 and ionic strength = 0.15, containing 0.05% (w/v) of Triton X-100, necessary to prevent adherence of the enzyme to the cuvette walls [21]. All solutions contained also 1.5% (v/v) (final concentration) of dimethyl sulfoxide, necessary to solubilize MR889 and the substrate. This concentration of dimethyl sulfoxide was not deleterious to the enzyme, as demonstrated by linear reaction rates for at least 15 min at different substrate concentrations. The progress of the reaction was followed fluorimetrically by measuring the appearance of 7-amino-4-methylcoumarin (λ_{ex} = 383 nm; λ_{em} = 455 nm) in thermostatted disposable acryl cuvettes (Sarstedt, Nümbrecht, F.R.G.) at 37° under continuous stirring [29]. The recorder scale was calibrated with 7-amino-4-methylcoumarin solutions of known concentration determined by using an absorption coefficient for this substance of 16,000 M^{-1} cm^{-1} at 342 nm. All solutions were made up with water doubly distilled from a quartz apparatus. The kinetic parameters k_{cat} and K_m were determined for the elastase substrate under the same experimental conditions used for inhibition studies in the substrate concentration range 0.1 to 1.6 mM and were: k_{cat} = 29.7 ± 0.6 sec^{-1}, K_m = 0.70 ± 0.04 mM, as determined by fitting the data by non-linear regression to the Henri–Michaelis–Menten equation. For substrate concentrations higher than about 2 mM substrate inhibition was observed.

Cathepsin G and α-chymotrypsin were assayed with Suc—Ala—Ala—Pro—Phe—*p*-nitroanilide [30] by following the release of *p*-nitroaniline at 410 nm in the same buffer system as for leukocyte elastase. Cathepsin B was assayed fluorimetrically with Z—Arg—Arg—NMec in 0.1 M Na^+/K^+ phosphate buffer, pH 6.00, containing 2 mM EDTA, 2 mM dithiothreitol and 1.5% (v/v) dimethylsulfoxide [31]. The elastolytic activity of leukocyte elastase was measured according to Schwabe [32]. Briefly, 2.0 mg of elastin from bovine neck ligament (Sigma) were incubated with enzyme with or without inhibitor in an end volume of 0.4 mL for 120 min at 37° using the same buffer system described for the continuous assay. The solution was then made 5% (w/v) with trichloroacetic acid and centrifuged. A 0.1-mL portion of the clear supernatant was added to 3.0 mL of 0.2 M sodium borate buffer, pH 8.5, followed by addition under vigorous stirring with 1.0 mL of fluorescamine solution (15 mg/100 mL in acetone). The fluorescence of the labelled peptides was monitored with λ_{ex} = 390 nm and λ_{em} = 480 nm. The fluorescence obtained in the absence of inhibitor was taken as reference (100% activity).

Apparatus. Fluorescence was monitored with an Aminco SPF-500 recording spectrofluorimeter operating in the ratio mode. For spectrophotometric measurements an Uvikon 810 apparatus was used.

Theory and treatment of the kinetic data. Progress curves were continuously monitored for several minutes and data points were sampled to obtain 30–40 points for each curve as shown in the example of Fig. 1 (curve with [*I*] = 2.4 μM). These data were fitted by non-linear regression to the following equation for slow-binding inhibition [33, 34]:

$$[P] = v_s t + (v_0 - v_s)(1 - e^{-k_{app}t})/k_{app} + d. \quad (1)$$

The symbols v_0, v_s and k_{app} represent the initial velocity, steady-state velocity and an apparent pseudo-first-order rate constant; *d* is a displacement term to account for the fact that at $t = 0$ the fluorescence may not be accurately known [35].

Progress curves for both of the mechanisms A and B shown below can be described by Eqn (1), and a distinction between them is possible by analysing the dependence of v_0 and k_{app} upon the inhibitor concentration: for mechanism A v_0 is independent of [*I*] and k_{app} is a linear function of [*I*], whereas for mechanism B v_0 decreases by increasing [*I*] and k_{app} is a hyperbolic function of [*I*] [See Eqns (2) and (3) below] [33, 34].

The expressions for the apparent, pseudo-first-order rate constants k_{app} that characterize mechanisms A and B are:

$$\text{Mechanism A: } k_{app} = \frac{k_{on}}{1+\sigma}[I] + k_{off} \quad (2)$$

$$\text{Mechanism B: } k_{app} = k_6 \left[\frac{1 + \dfrac{[I]}{K_i^*(1+\sigma)}}{1 + \dfrac{[I]}{K_i(1+\sigma)}} \right] \quad (3)$$

where $\sigma = [S]/K_m$. The significance of the equilibrium constants in Eqn (3) is as follows: $K_i = k_4/k_3$; $K_i^* = K_i[k_6/(k_5 + k_6)]$.

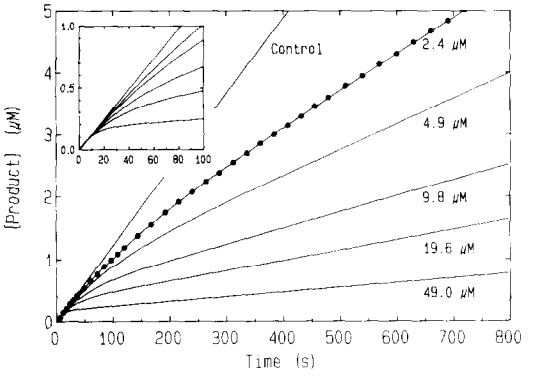
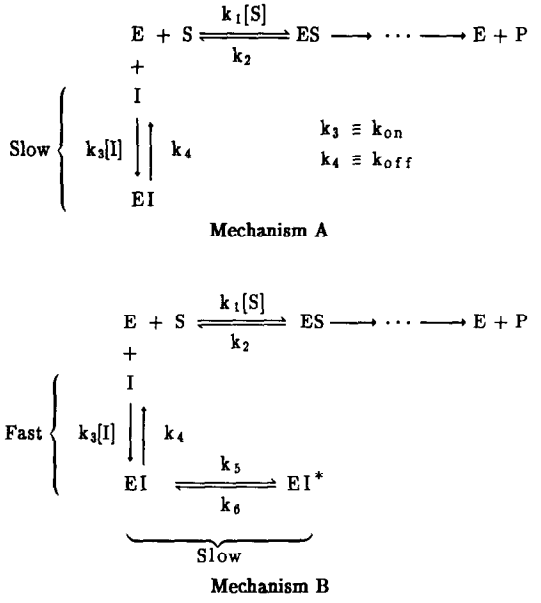


Fig. 1. Progress curves for the inhibition of leukocyte elastase by MR889. The experimental points sampled from the progress curves are shown only for the curve with [*I*] = 2.4 μM to illustrate the method. The continuous lines are best fit values of data to Eqn (1). The inset shows an enlargement of the lower left part of the diagram in order to show the independence of the initial velocity upon the inhibitor concentration. The substrate was MeO—Suc—Ala—Ala—Pro—Val—NMec at a concentration of 0.20 mM: $[E]_t$ = 2.4 nM; the numbers in the diagram indicate MR889 concentrations. Na^+/K^+ phosphate buffer (57 mM), pH 7.40, containing 0.05% (w/v) of Triton X-100 and 1.5% (v/v) dimethyl sulfoxide; temperature = 37°. The reaction was started by adding enzyme to preincubated substrate and inhibitor.



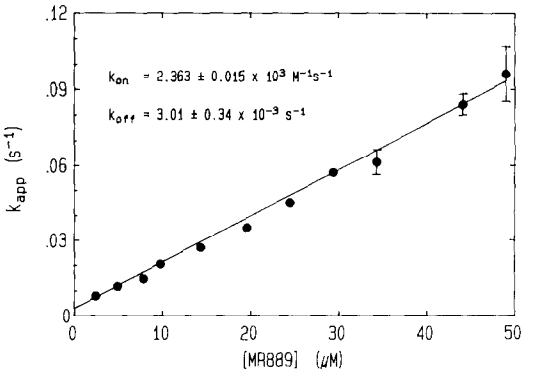


Fig. 3. Plot of k_{app} vs MR889 concentrations. The apparent rate constants (k_{app}) were calculated from experiments as shown in Fig. 1 for various inhibitor concentrations, weighted according to the squared inverse of their standard errors and fitted to Eqn (2) to obtain k_{on} and k_{off} shown in the diagram. The standard errors of the first eight points are within the size of the symbols.

The values of k_{app} obtained by non-linear regression of experimental data using Eqn (1) were weighted according to the squared inverse of their standard errors and fitted to Eqns (2) and (3) to obtain the values of the microscopic rate constants and of the equilibrium inhibition constant. Calculations were performed using the Enzfitter software package [36].

RESULTS

Pre-steady-state kinetic analysis

Reaction progress curves were obtained using a fixed human leukocyte elastase concentration (2.4 nM of titrated active sites) at three concentrations of the fluorogenic substrate MeO—Suc—Ala—Ala—Pro—Val—NMec and several concentrations of MR889. Some reaction profiles are shown in Figs 1 and 2. When the reaction was started by adding enzyme to a solution containing substrate and inhibitor the reaction profiles were characterized by a concave upward lag phase, corresponding to an exponential transient state, followed by a linear steady-state. The apparent rate constant and the amplitude of the transient as well as the steady-state slope depended on the inhibitor concentration. As it can be seen in Figs 1 and 2 (see in particular the inset in Fig. 1) the velocity at zero time (v_0) was independent of the inhibitor concentration at all of the three substrate concentrations used. The values of the apparent pseudo-first-order rate constants, k_{app}, obtained by non-linear regression of the experiments shown in Fig. 1 and six additional experiments were linearly dependent on the inhibitor concentration (Fig. 3), thus the best fit of k_{app} vs [I] was obtained using Eqn (2) instead of Eqn (3). From the slope and intercept of this plot k_{on} and k_{off} were calculated according to Eqn (2): $k_{on} = 2363 \pm 15\ M^{-1}\,sec^{-1}$, $k_{off} = 3.01 \pm 0.34 \times 10^{-3}\,sec^{-1}$, from which $K_i = k_{off}/k_{on} = 1.27 \pm 0.15\ \mu M$. The standard error of the quotient k_{off}/k_{on} was calculated with the formula given by Morrison and Uhr [37].

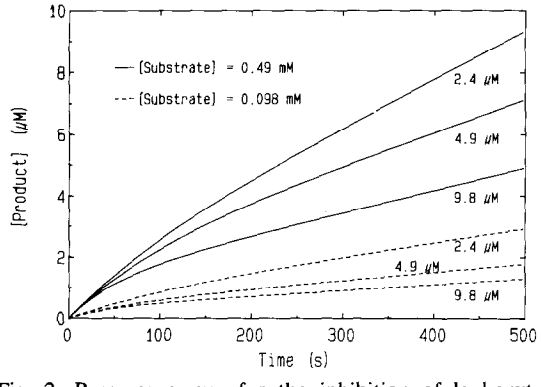


Fig. 2. Progress curves for the inhibition of leukocyte elastase by MR889. Conditions as in Fig. 1, at the two substrate concentrations indicated.

The same steady-state slopes shown in Figs 1 and 2 were also obtained when the reactions were started by adding substrate to solutions in which enzyme and inhibitor (at the same concentrations as in Figs 1 and 2) had been preincubated for 15–20 min. In this case the concave downwards lag phase was absent and the reaction profiles were linear from the beginning (not shown). The expected concave upwards lag phase, characteristic of slow-binding inhibition [33, 37], was too short to be observed with accuracy. These experiments demonstrate the full reversibility of the interaction between MR889 and elastase.

Steady-state kinetic analysis

The slopes of the linear parts of the kinetic profiles shown in Figs 1 and 2 were used for analysing the type of inhibition and for calculating the inhibition equilibrium constant by means of the specific velocity plot [38]. This particular plot was chosen because it

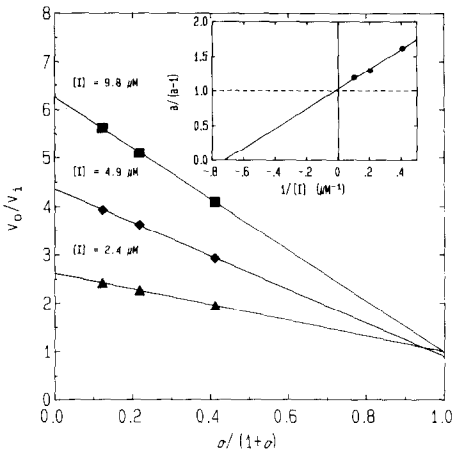


Fig. 4. Specific velocity plot of the steady-state slopes of the curves in Figs 1 and 2. $\sigma = [S]/K_m$. The inset shows a replot in which a represents the intercept of the straight lines in the primary plot with the v_0/v_i axis for $\sigma/(1 + \sigma) = 0$.

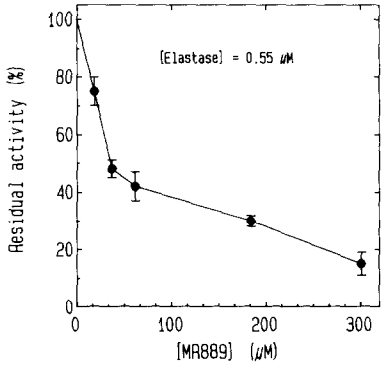


Fig. 5. Inhibition profile of leukocyte elastase by MR889 using insoluble bovine neck ligament elastin as the substrate.

can easily distinguish linear and hyperbolic inhibition and at the same time it offers a clear graphic demonstration of the inhibition type (competitive, non-competitive, uncompetitive or mixed). In Fig. 4 the three straight lines, obtained at three substrate and three inhibitor concentrations, converge to a common point with coordinates 1/1. The secondary plot shown in the inset of Fig. 4 intersects the ordinate axis at 1.0. These features are typical of a reversible, fully (linearly) competitive inhibition mechanism [38]. The value of the inhibition equilibrium constant obtained by the graphic analysis shown in Fig. 4 was $K_i = 1.38$ μM, in excellent agreement with the result obtained by pre-steady-state analysis.

Effect of MR889 on the elastolytic activity of leukocyte elastase

When using insoluble elastin as the substrate of human leukocyte elastase, much higher concentrations of enzyme than those used in steady-state experiments are required in order to gather sufficient reaction products for a precise quantitative analysis. In the experiment shown in Fig. 5 the enzyme was present at a concentration of 0.55 μM, i.e. at a concentration 220 times larger than that used in the experiments of Figs 1–4. It is thus clear that also higher concentrations of inhibitor must be used. The aim of the experiment in Fig. 5 is to show that MR889 is also able to inhibit human leukocyte elastase in the presence of a natural, insoluble, macromolecular substrate.

Effect of MR889 on the activity of α-chymotrypsin, cathepsin G and cathepsin B

The activity of these three enzymes was not affected by MR889 up to a concentration of 0.1 mM. Even for prolonged incubation times there was no decrease of the activity compared with the controls.

DISCUSSION

The inhibition mechanism of human leukocyte elastase by the cyclic thiolic compound MR889 can be classified as reversible, slow-binding, fully competitive according to Mechanism A shown in Materials and Methods, for which Eqns (1) and (2) apply [34]. The salient features of this mechanism can be summarized as follows: (i) the steady-state is attained at a slow rate on the steady-state time scale when the reaction is started by adding enzyme to a solution containing substrate and inhibitor; (ii) the velocity at zero time (v_0) is independent on inhibitor concentration; (iii) the apparent pseudo-first-order rate constant of the exponential phase of the reaction depends linearly upon the inhibitor concentration; (iv) the degree of inhibition depends on both the inhibitor and substrate concentration, with decreasing inhibitory power for increasing $[S]$ at a given $[I]$. It could be questioned whether Mechanism B would be a more adequate reaction pathway for describing the observed inhibition. However, as pointed out by Morrison [34], the possibility of distinguishing between Mechanisms A and B depends on the relative magnitudes of K_i and K_i^*. If it happens that $K_i \gg K_i^*$ and the inhibitor is varied in the region of its K_i^*, then Mechanism B degenerates into Mechanism A [34]. Thus, for the inhibition of human leukocyte elastase by MR889, Mechanism A or an equivalent "degenerated" Mechanism B is valid. Also the nature of the interaction between MR889 and leukocyte elastase at the molecular level, remains obscure at present. In fact, this kind of information is not accessible when only kinetic studies are performed. The knowledge of the complete mechanism of action of an inhibitor requires, besides kinetics, an implementation by other studies, including protein modifications, three-dimensional structures, NMR and others. For instance, in this study we did not consider the possibility that tautomeric forms of MR889 may exist in aqueous solution or that enantiomers and diastereoisomers of this molecule may contribute in different ways to inhibition. However, studies with pancreatic elastase and insoluble elastin as substrate have demonstrated that MR889 and its isolated diastereoisomers inhibit the enzyme with almost the same efficiency (L. Galzigna, authorized personal communication). In some particular circumstances it may happen that a slow-binding process is not really due to a slow encounter

of the reactants, but to the co-existence of two or more forms of the inhibitor in solution. For instance, it has been shown that the peptide aldehyde leupeptin, an inhibitor of serine and cysteine endopeptidases including cathepsin B [29], exists in solution in three equilibrium forms: as hydrate, as cyclic carbinolamine and as free aldehyde [39]. The aldehyde form, which is the only one responsible for enzyme inhibition, is present at a very low concentration. For this reason, despite a high k_{on} value, the observed rate of complex formation between leupeptin and cathepsin B, $k_{app} = k_{on}[I] + k_{off}$, is slow [39]. With these concepts in mind it would be better to simply call "slow inhibition" what is generally called "slow-binding inhibition" [34, 40] and to consider the slow process of complex formation in a purely kinetic sense, meaning that the experimentally observable formation of the enzyme–inhibitor complex is slow on the steady-state time scale. Independently of the real mechanism of action, kinetic studies are always an indispensable tool for measuring the "practical" constants of interaction between enzymes and inhibitors, which in turn are useful for predicting the possible physiological significance of the inhibition.

The inhibitory action of MR889 on leukocyte elastase could also be demonstrated using insoluble elastin as the substrate, thus confirming that the inhibition is not restricted to "artificial" systems employing synthetic peptide substrates.

MR889 does not inhibit α-chymotrypsin and cathepsin G (serine endopeptidases) nor cathepsin B (a cysteine endopeptidase), but inhibits porcine pancreatic elastase (data not shown, obtained under the same conditions as for leukocyte elastase). Although other enzymes were not taken into account, α-chymotrypsin, cathepsin G and cathepsin B are typical representatives of a large class of enzymes with which the inhibitor may in principle interact, so that it can be concluded that MR889 recognizes preferentially elastase-type endopeptidases.

It has been pointed out that the property of inhibiting extracellular matrix-degrading endopeptidases in the presence of their natural, insoluble substrates is a necessary prerequisite for the practical use of inhibitors of this category of enzymes. The final decision of whether an inhibitor of endopeptidases can be used in practice or not will further depend on the interplay between specificity, efficiency and other properties of the inhibiting substance [41]. In particular, a non-toxic inhibitor with a relatively modest efficiency will probably be superior to a very efficient, but toxic compound. The toxicity of MR889 was investigated in mice and rats after oral administration (dosage up to 5 g/kg body wt) or intraperitoneal administration (dosage up to 2 g/kg body wt). No mortality at all was observed in the two species using the two routes of administration (L. Galzigna, authorized personal communication). The emphysematous process consists of a progressive and irreversible degradation of the lung elastic tissue and occurs when the physiological balance between the pulmonary leukocyte elastase activity and that of its endogenous inhibitor α1-proteinase inhibitor is altered [11, 12]. Although MR889 is characterized by a relatively low efficiency towards elastases when compared with other compounds [15–17], its high specificity for elastolytic enzymes and the lack of toxicity may constitute a rational basis for its use in the treatment of emphysema.

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