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Reversible, Slow, Tight-Binding Inhibition of Human Leukocyte Elastase

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CBz-Ala-Ala-Pro-ambo-Val-CF_3 (1) was synthesized. The compound inhibits human Leucocyte elastase with K_i = 1.0 x 10^{-9} M. This inhibitor is reversible, slow, tight-binding inhibitor with k_{on} = 2 x 10^4 M^{-1} s^{-1} k_{off} = 1.9 x 10^{-5} s^{-1}. For the solubilization of elastin by HLE by 1 $I.C._{50}$ = 110 nM. This inhibitor is the most effective aldehyde or ketone inhibitor of a serine proteinase yet described.

Peptidyl trifluoromethyl ketones are effective transition-state analogues (1) of a variety of hydrolytic enzymes (2), including the serine proteinases, α-chymotrypsin and porcine pancreatic elastase, PPE, (3). Human leukocyte elastase, HLE is a serine proteinase which has been implicated in emphysema (4), adult respiratory distress syndrome (5) and rheumatoid arthritis (6). The inhibition of HLE has therefore been the subject of several studies. Peptidyl aldehydes (7) and peptidyl boronic acids (8) are reversible inhibitors. The best K_i of each class are 80 and 0.6 nM, respectively. Of the suicide inhibitors only the cephalosporins (9) appear to have sufficient hydrolytic stability to be of interest for

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The abbreviated designation of the peptidyl inhibitors is as defined in the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1985). The term ambo designates the residue which follows as being racemic. Other abbreviations used are as follows: Cbz, benzyloxy carbonyl; Succ, succinyl; MeOSucc, methoxysuccinyl; AAPV, L-alanyl-L-alanyl-L-prolyl-L-valinyl; AMC, 4-amino-7-methyl courmarin; pNA, p-nitro aniline; PPE, porcine pancreatic elastase; HLE, human leukocyte elastase.

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therapeutic effect. We now explore the efficacy of a trifluoromethyl-ketones as inhibitors of HLE.

METHODS

Enzymes and Enzyme Assays - For the assay with insoluble elastin, HLE was purified from sputum (10), characterized and found to be 98% active (11), as determined by active site titration with p-nitrophenyl-3-(N-acetyl-L-alanyl-L-alanyl)-2-methyl carbazate, an azapeptide compound that was kindly provided by Dr. James C. Powers, Georgia Institute of Technology, Atlanta, GA (12).

HLE† was assayed by three procedures. The first monitored the increase in absorbance at 375 nm using 40 nM HLE and SuccAAPV-AMC (0.4 to 2.0 mM) as substrate in pH 7.80 HEPES (50 nM) with 0.1 M sodium chloride. The second procedure used 1 to 10 nM HLE and MeOSuccAAPV-pNA (0.02 mM to 0.5 mM) as substrate in pH 7.80 HEPES (50 mM) with 0.5 M sodium chloride and 1 mg/mL bovine serum albumin. The increase in absorbance at 410 nm was monitored. Substrates and inhibitors were dissolved in DMSO or ethanol as necessary. The organic solvent in the assays did not exceed 3% v/v by the first method and 1% v/v by the second method. All assays were determined at 25°C in UV/vis spectrophotometers.

The activity of HLE was also assayed with an insoluble elastin substrate, ^{3}H-calf ligamentum nuchae elastin, as previously described with the following modifications (13). Each assay tube contained HLE (86 nM), 5 mg elastin, and 2 mg bovine serum albumin in 4 ml of phosphate buffered saline, pH 7.5, in the absence of sodium dodecyl sulfate. The buffer containing albumin had been filtered with a 0.22 um filter. Inhibitor was added to assay tubes followed by HLE within 1 min. HLE was also added to 3 tubes without inhibitor. Other tubes received neither HLE nor inhibitor. The effect of inhibitor on the background activity was also determined and found to be negligible. All assay tubes were incubated at 37°C for 1 or 4 hr followed by centrifugation and filtration. The filtrate was assessed for tritium in a liquid scintillation spectrometer. Results were corrected for the blank values (around 4% of values achieved with HLE) and expressed as % activity (HLE in the absence of inhibitor = 100%). The inhibitor concentration resulting in 50% activity, $I.C._{50}$, was derived by interpolation of the plot of % activity vs. concentration of inhibitor (least squares analysis).

Determination of Kinetic Constants. The association rate constant (k_{on}) were determined by solving equation 1

$$k_{obs} = k_{off} + k_{on}[I]/(1 + [S]/K_m) \qquad (1)$$

(14) where k_{obs} is determined from a progress curve (15). Additionally, k_{on} was determined by incubating enzyme and inhibitor for various times and determining remaining enzyme concentration under pseudo-first-order conditions where the inhibitor concentration was > 60 times the enzyme concentration. Dissociation rate constants (k_{off}) were determined by preforming the enzyme-inhibitor complex, diluting 200 fold into buffer and at various times, over 16 hours, measuring the return of activity by adding substrate to an aliquot and measuring initial rates. Controls demonstrate that there is no detectable loss of enzyme activity under the conditions of the second assay procedure over an 18 hour period. K_i was determined by solving

† HLE, Succ-AAPV-AMC, and Adamantyl Sulfinyl Chloride, were kind gifts of Dr. T. Payne of Sandoz Ltd.

equation 1 at a series of inhibitor concentrations where v_s is the inhibited, steady-state.

$$v_s/v_o = (1 + [S]/K_m)/(1 + [S]/K_m - [I]/K_i)$$

velocity and v_o is the uninhibited velocity as described by Cha (15).

RESULTS and DISCUSSION

Cbz-Ala-Ala-Pro-ambo-Val-CF_3 (1) was synthesized† essentially by a previously described procedure for similar peptides (16). 1 has the same amino acid sequence as the substrate with the maximal V/K of a series of substrates (17). It has been pointed out that for transition state analogs, K_i of the inhibitor varies proportionally with V/K of the corresponding substrate (18, 19). We tested 1 as inhibitor against HLE and determined K_i = 1.1 nM. Inhibitor 1 is the most effective inhibitor, based on a ketonic or aldehydic peptides. described so far, for HLE. Its K_i is close to that for eglin (K_i = 8 x 10^{-10} M) (20), a naturally occurring protein inhibitor.

Inhibitor 1 exhibited a lag time before the establishment of steady state velocity. The k_{on} as determined by the method of Cha (15) and by the preincubation method (see methods) gave similar results of 1.6 and 2.0 x 10^4 M^{-1} s^{-1}. respectively. The determination of k_{off} by two different methods gave similar results. Multiplying K_i (1.1 nM) by the directly determined k_{on} (2 x 10^4 M^{-1} sec^{-1}) results in a k_{off} of 2.2 x 10^{-5} s^{-1}. The value of k_{obs} obtained from the rate of recovery of HLE activity upon dilution (200 x), is 5.5 x 10^{-5} s^{-1} with a total recovery of catalytic active of 35%. The observed rate for equation 2 is $k_{obs}=[E-I]*k_{off}+[E]*[I]*k_{on}$. Since the initial inhibitor concentration was only slightly larger than the initial HLE concentration, a TUTSIM model of equation 2 was used to determine k_{off}. Using the independently determined k_{on} (1.8 x 10^4

† A procedure giving details of synthesis and pertinent NMR data will be supplied upon request to R.H.A. Peptide 1 gave a single spot on TLC (R_f 0.5, 10% MeOH in $CHCL_3$), and acceptable elementary analysis (C,H,N) as well as mass-spectrum.

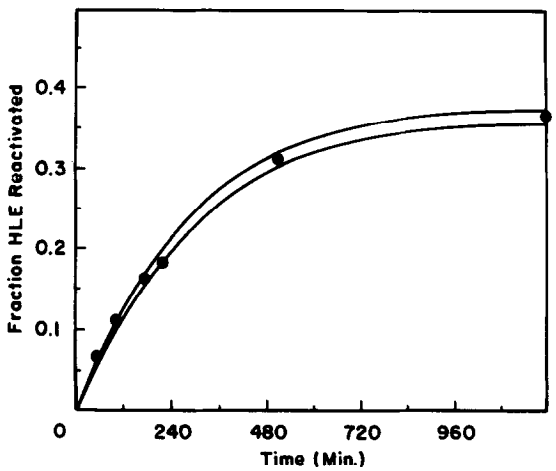

FIGURE 1. Reactivation of HLE after dilution. HLE (.44 μM) and 1 (0.60 μM) were diluted to 2.2 nM and 3.0 nM, respectively. At indicated times 0.50 mM MeOSucc-AAPV-pNA added and free HLE (O) determined. Upper curve is calculated for k_{off} of 2.0 x 10^{-5} and lower curve is calculated for 1.8 x 10^{-5} s^{-1}, experimental points.

M^{-1} s^{-1}) it is shown in Figure 1 that values of k_{off} of 1.8 to 2.0 x 10^{-5} s^{-1} bracket the observed data.

$$[\text{Enzyme - Inhibitor}] \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} [\text{Enzyme}] + [\text{Inhibitor}] \qquad (2)$$

These results give a K_i of 0.9 nM in excellent agreement with the K_i (1.1 nM) determined by equation 1.

The inhibitory effect of 1 upon the hydrolysis of elastin by HLE was also examined. This assay may be more representative of in vivo conditions. Assays were carried out for one hour and four hour periods. For the one and four hour assay $I.C._{50}$ values for 1 were 110 and 120 nM respectively. The $I.C._{50}$ of inhibitor 1 compares favorably with those for Succ-AAPV chloromethyl ketone and Succ-AAP-ambo-boro-Val pinacol of 292 nM and 130 nM, respectively (unpublished data), but are not as effective on a molar basis as the naturally occurring inhibitors alpha-1-protease inhibitor (60 nM) (21) and eglin c (43 nM) (11).

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