J. Enzyme Inhibition, 1997, Vol. 11, pp. 183–198
Reprints available directly from the publisher
Photocopying permitted by license only

SYNTHETIC MACROMOLECULAR INHIBITORS OF HUMAN LEUKOCYTE ELASTASE. 2. EFFECT OF LOADING OF A PEPTIDYL CARBAMATE INHIBITOR AND MOLECULAR SIZE OF POLYMER BACKBONE ON ITS INHIBITORY CAPACITY

FATEMEH MOHAMMADI, DAGMAR NOSEK and GEORGE A. DIGENIS*

Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082, USA

(Received 8 March 1996)

Several macromolecular inhibitors of human leukocyte elastase (HLE) were prepared by covalently bonding a low molecular weight HLE inhibitor peptidyl carbamate, *p*-nitrophenyl-*N*-[succinyl-L-alanyl-L-ananyl-L-prolylmethyl]-*N*-isopropyl carbamate **1**, with the neutral hydrophilic polymer, poly-α,β-[*N*-(2-hydroxyethyl)-D,L-aspartamide], PHEA **2**. These novel polymeric compounds differed in the molecular size of their PHEA polymer backbone and the extent of loading of the peptidyl carbamate, (PC). They were shown to efficiently inhibit HLE (K_i = 97 to 12.8 nM) as intact macromolecular entities and were found to be more stable to hydrolysis than the non-polymer bound low molecular weight inhibitor **1**.

The inhibition of HLE by the novel macromolecular inhibitors was found to be noncompetitive and reversible, proceeding via slow formation of inhibitor-enzyme complex. The effect of loading of **1** and molecular size of the PHEA **2** polymer on enzymatic parameters K_i, k_{on} and k_{off} is discussed and a possible mechanism of inhibition is presented.

Keywords: Human leukocyte elastase (HLE); peptidyl carbamate; *p*-nitrophenyl-*N*-[succinyl-L-alanyl-L-alanyl-L-prolylmethyl]-*N*-isopropylcarbamate; enzyme-inhibitor complex; inhibitory capacity; poly-α,β[*N*-(2-hydroxyethyl)-D,L-aspartamide]; PHEA; polysuccinimide; effect of loading; molecular size; polymeric inhibitor; mechanism of inhibition.

INTRODUCTION

Inhibitors of Human Leukoctye Elastase (HLE) have been under investigation for many years. An excess of active HLE has been implicated in various pathological states associated with the abnormal degradation of connective tissue, such as rheumatoid arthritis,[1] adult respiratory distress syndrome (ARDS),[2] and pulmonary emphysema.[3] In these and similar conditions the most evident pathogenesis

*Correspondence. (606)257-1970.

is proteinase-antiproteinase imbalance.[4] It is generally believed that pulmonary administration of an exogenous inhibitor could reduce proteinase activity, thus restoring equilibrium and protecting the lungs from development of emphysema and related diseases.[3]

The two types of HLE inhibitors that have been investigated for pulmonary administration are natural inhibitors (for replacement therapy)[4] and synthetic compounds of low molecular weight. Synthetic elastase inhibitors reported are either irreversible or reversible inhibitors of HLE.[5–9] Research in our laboratories has focused on reversible inhibitors of the peptidyl carbamate type.[10–16] These low molecular weight derivatives were designed to react with the active site of HLE. In spite of high *in vitro*, inhibitory potency the therapeutic efficacy of these peptidyl carbamates has been largely offset by a short biological half-life, which can be attributed to either rapid elimination from the target compartment lung or to metabolic degradation.[17]

In order to prolong the biological half-lives of peptidyl carbamates, a macromolecular derivative was designed. It has been hypothesized that in certain applications, such as in the control of emphysema[3] and arthritis,[1] a synthetic macromolecular inhibitor should exhibit a prolonged residence time in the lung due to the slower clearance or greater *in vivo* stability.[14] Slower clearance of a low molecular weight inhibitor from the targeted compartment should therefore be realized by covalently linking of the inhibitor to a hydrophilic polymer, provided that activity against HLE is not compromised. Additionally greater *in vivo* stability may result from the presence of polymer, which may protect the inhibitory moiety from metabolic degradation.

We have previously reported that coupling of peptidyl carbamate (PC) **1** with synthetic poly peptide, PHEA **2**, resulted in a potent and selective HLE inhibitor.[14] This polymer-bound PC was shown to protect the hamster lung from HLE induced emphysema, when administered intratracheally. Furthermore, after coupling with polymer, the inhibitors residence time in the lung was significantly increased from 4 minutes to 421 minutes. Therefore compounds of this class were selected for further development and investigation.

A series of polymeric derivatives of **1** was designed and synthesized by altering the molecular size of the polymer carrier (PHEA) and the extent of loading of the inhibitory moiety **1**. The resulting macromolecular peptidyl carbamates were studied for their *in vitro* enzymatic activity and mechanism of inhibition.

MATERIALS AND METHODS

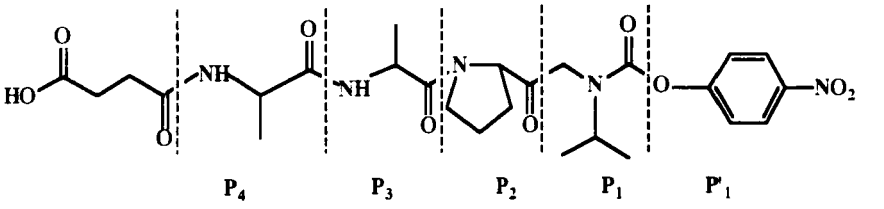
Microanalyses were performed by Atlantic Micro Lab Inc., Atlanta, Georgia, and were within ±0.4% of theoretical values. Amino acid analyses were performed by

the Cancer Research Center at the University of Kentucky. Infrared (IR) spectra were determined as Nujol mulls or liquid films on a Perkin-Elmer Model 1430 ratio recording spectrophotometer. Reactions were routinely followed by thin layer chromatography (TLC) using Whatman K6F silica gel plates. Spots were visualized by UV light (254 nm) or exposure to iodine vapor. Gel permeation chromatography (GPC) was carried out on three columns of different size and packing, employing a Waters 650 Advanced Protein Purification System interfaced with a Waters 481 LC spectrophotometer and Waters 740 Data Module. Nuclear magnetic resonance (NMR) spectra were recorded using either a Varian Gemini 200 MHz or VXR-400 spectrometer with tetramethylsilane as an internal standard. Ultraviolet and visible spectral measurements were executed on a Cary model 2200 UV-Visible spectrophotometer. The HPLC system used was an ABI Model 400 solvent metering pump (Applied Biosystems Inc), Model 878A autosampler, Model 783 UV absorbance detector, and 429A Integrator. The C-18 columns (25 × 0.46 cm, 10 micron) were purchased from Whatman.

Preparation of PC 1 and Polymer Carrier PHEA 2

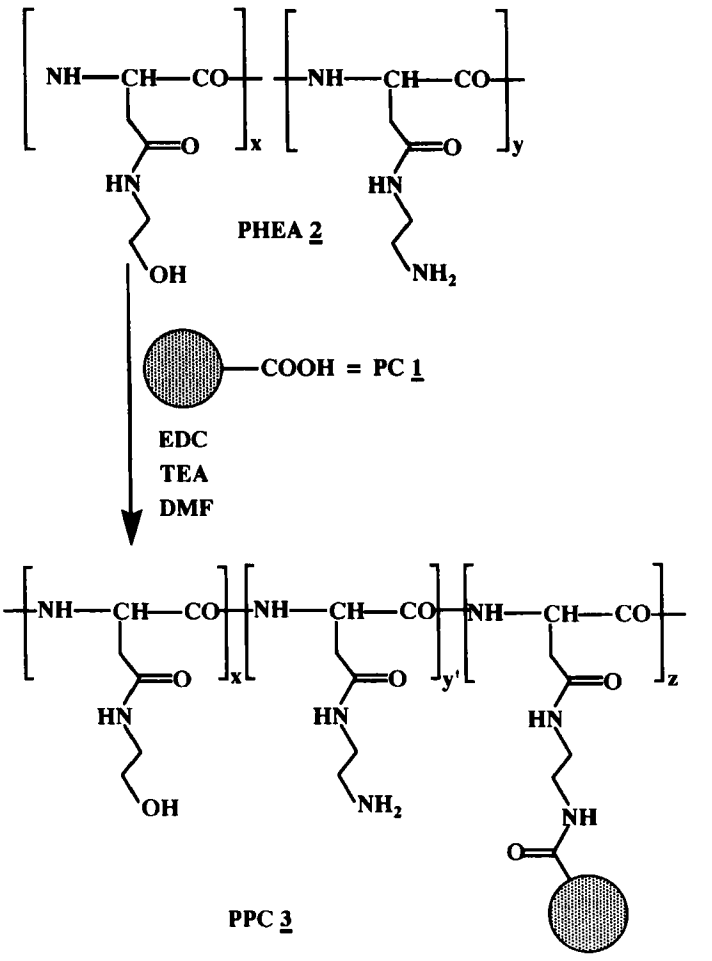
PC **1** (Figure 1) was synthesized according to a previously published procedure.[12] Polymer carrier PHEA **9** prepared by reaction of poly D,L-succinimide with ethanolamine and mon-BOC diaminoethane according to previously developed procedure.[18,19]

The desired average molecular weights (27, 17 and 7 kDa) of PHEA were obtained using room temperature and ethanolamine polysuccinimide ratio of 2:1. Under these mild conditions the reaction was found to be completed after seven days.



PC 1

FIGURE 1 The general structure of *p*-nitrophenyl-*N*-[succinyl-L-alanyl-L-alanyl-L-prolylmethyl]-*N*-isopropyl carbamate PC1, (HLE inhibitor of the carbamate type). $P_4 \ldots P'_1$ are residues which are known to interact at or near the active site, $S_4 \ldots S'_1$, of the enzyme.



SCHEME 1

Polymer-bound Inhibitors

The macromolecular peptidyl carbamate PPC **3**, (Scheme 1) was prepared by the coupling reaction of **2** with the peptidyl carbamate **1** according to our previously described methods.[14] However, the degree of loading of PC **1** on the polymer HEA **2** was controlled during the synthesis by concommitment utilization of our previously developed GPC analytical method.[16]

Analyses

The stability of **1** and **3** (Scheme 1) were assessed at pH 8.8 in a 0.01 M phosphate buffer solution containing 0.15 m NaCl. The *in vitro* half-lives of **1** and **3** were determined from *p*-nitrophenol release at 400 nm. The number of amino groups in the polymer **2** and polymer-bound inhibitor **3** was determined spectrophotometrically at 430 nm from derivatization with 2,4,6-trinitrobenzene sulphonic acid (TNBS).[20] The number of peptidyl carbamate units per mole of polymer inhibitor (the loading) was determined at 276 nm (λ_{max} of **1**) by using the molar absorptivity of the unbound inhibitor **1** (ϵPC = 9800).

Separation of the polymer-bound inhibitor and free inhibitor was performed by GPC on a Sephadex G-25 Superfine (11 × 140 mm) column. The mobile phase consisted of 0.01 M phosphate buffer at pH 8.8 containing 0.15 M NaCl, with a flow rate of 1 mL/min. The absorption of the eluate was monitored at 276 nm. This separation technique was applied to follow the time course of the coupling reaction between **1** and the polymer **2**. The column was also employed to analyze the composition of the enzymatic mixture during the mechanistic study of the enzyme-inhibitor (E-I) interaction. The values of molecular weight and number molecular weight averages (M_w and M_n) polydispersity (pd), and molecular weight distribution profiles of all the polymer samples were determined by GPC using broad molecular weight standards (authentic samples of PHEA, kindly provided by Dr. Frantisek Rypacek, Institute of Macromolecular Chemistry, Prague, Czech Republic). The molecular weights of standard samples were obtained independently from light scattering measurements (Institute of Macromolecular Chemistry, Prague, Czech Republic). The analysis of the starting polymers **2** and polymer-bound inhibitors **3** were preformed on a mixed bed column (13 × 410 mm Sepharose CL-4B, Sephacryl S-200, Sephadex G-25 Superfine in a volume ratio of 16:5:3, respectively). The eluent was a solution of 0.005 M phosphate buffer containing 0.15 M NaCl at a flow rate of 0.2 mL/min The absorption of the eluate was monitored at 220 nm for **2** and 276 nm for **3**. In a typical example, 5 mg of the sample was dissolved in 1 mL of the eluent buffer (0.5%, w/v) and the volume applied to the column was 100-200 μL. GPC data were treated with the curve summation method.[21] The column calibration was performed with a series of polydisperse fraction of PHEA with M_w 33, 63 and 143 kDa, respectively.

Enzyme Assays

HLE, its synthetic substrate (methoxysuccinyl-L-alanyl-L-prolyl-L-valine-*p*-nitroanilide), and Hepes, [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] were obtained from Sigma Chemical Company. All enzyme assays were performed

spectrophotometrically at 25°C using a Varian 2200 Cary spectrophotometer. The activity of HLE was measured using the synthetic substrate and monitoring the rate of production of *p*-nitroanilide at $\lambda = 410$ nm.[22]

Screening for Inhibitory Activity

In a typical experiment, the inhibitor (0.05 mL, 5 μL–10 nM in Hepes buffer) and the substrate (0.05 mL, 27 mL in DMSO) were added to 2.9 mL of Hepes buffer pH 7.5 in two quartz cuvettes, thermally equilibrated in the spectrophotometer for 2 min. The cuvettes were shaken for 20 s, and the absorbance was balanced at 410 nm. At t=0, the HLE (0.005 mL, 27 μM in 0.5 M acetate buffer, pH 5.5) was placed into the sample cuvette and acetate buffer (0.005 mL, pH 5.5) was added to the reference cuvette. The mixture was shaken for 20 s, and the increase in absorbance was monitored for 30 min. In the control experiment, 0.05 mL of Hepes buffer was substituted for the inhibitor solution to establish 100% enzyme activity. Inhibitory activity was assumed present if the rate of substrate hydrolysis was diminished by a minimum of 10%. Initial velocities were determined from a plot of absorbance versus time.

Kinetic Studies — Steady State Study

The procedure described in the screening experiment was repeated using four different substrate concentrations and five different inhibitor concentrations. The absorbance data collected was used in Dixon[23] and Lineweaver-Burk[24] plots. In a typical experiment, the inhibitor (0.05 mL, 24–216 μM in Hepes buffer, pH 7.5) and the substrate (0.05 mL, 8.4–30 mM in DMSO) were added to 2.9 mL of Hepes buffer in two quartz cuvettes, thermally equilibrated in the spectrophotometer for 2 min. The cuvettes were shaken for 20 s, placed in the spectrophotometer and the absorbance was balanced at 410 nm. At t=0, the enzyme (0.005 mL, 37 μM in 0.5 M acetate buffer, pH 5.5) was placed in the sample cuvette and acetate buffer (0.005 mL) was placed in the reference cuvette. The mixture was shaken for 20 s, placed in the spectrophotometer and the increase in absorbance was monitored for 5 min. In a control experiment, Hepes buffer (0.05 mL was substituted for the inhibitor solution to establish 100% enzyme activity. The initial velocity was recorded and the Lineweaver-Burk plot (the reciprocal of velocity versus the reciprocal of substrate at various inhibitor concentrations) was constructed.[24] A Dixon plot, the reciprocal of velocity versus inhibitor concentration at various substrate concentrations, was also obtained from the initial velocities.[23]

Kinetic Studies — Bieth Method

This experiment was used to determine the K_i value and the IC_{50} value for the inhibitor. Substrate, buffer and enzyme concentrations were the same as used in the screening experiment. Inhibitor concentrations ranging from 0.9 μM to 28 μM were used.

Determination of Incubation Time

This experiment was carried out using a very low inhibitor concentration (80-90% of enzyme activity remaining) to follow the change of inhibitory activity with increasing incubation time. In a typical experiment, 0.05 mL of inhibitor was added to 2.9 mL of Hepes buffer in two quartz cuvettes. After equilibrating in the spectrophotometer and balancing the absorbance, a 0.005 mL aliquot of enzyme was placed in each of two quartz cuvettes. After shaking the cuvettes or 20 s, the enzymatic mixture was incubated for various time intervals (5–40 min). At the end of the incubation period, the absorbance was balanced at 410 nm and substrate (0.05 mL) was placed into both cuvettes. The mixture was shaken for 20 s and the increase in absorbance was recorded for 3 min. In a control experiment, Hepes buffer (0.05 mL) was substituted for the inhibitor solution to establish 100% enzyme activity. The percentage of remaining enzyme activity was then plotted against incubation time for each inhibitor concentration. An adequate incubation period was chosen as the shortest incubation time necessary for the lowest percentage remaining enzymatic activity.[25]

Determination of the K_i Value

In a typical experiment, 0.05 mL of inhibitor was added to 2.9 mL Hepes buffer in two thermally equilibrated quartz cuvettes and shaken for 20 s. At t=0, the enzyme (0.005 mL, 37 μM) was placed in the sample cuvette and 0.005 mL acetate buffer was placed in the reference cuvette. The enzyme mixture was shaken for 20 s, and then incubated for a predetermined period of time. At the end of the incubation period, the absorbance was balanced at 410 nm and substrate solution 0.05 mL was placed into both cuvettes. The mixture was shaken for 20 s, and the increase in absorbance was recorded for 3 min. In a control experiment, 0.05 mL of Hepes buffer was substituted for the inhibitor solution and the value thus obtained was assigned at 100% enzyme activity. The percent remaining activity of enzyme-inhibitor solution was then plotted against inhibitor concentration. This incubation curve was converted to an Easson-Stedman plot[26] the slope of which was chosen as the most accurate means of determining the K_i value in this series of experiments. The

inhibitor concentration necessary for 50% enzyme inhibition represented the IC_{50} value.

Determination of the Association Rate, k_{on}

Substrate, buffer and enzyme concentrations were the same as in the screening experiment. The inhibitor concentration used was equimolar to the enzyme concentration.

These experiments were conducted similar to those in the incubation time experiment. Inhibitor (0.05 mL) was added to 2.9 mL of Hepes buffer in two quartz cuvettes. After equilibrating in the spectrophotometer and balancing the absorbance, 0.005 mL of enzyme solution was placed into the sample cuvette and 0.05 mL Hepes buffer was placed into the referenee cuvette. After shaking the cuvettes for 20 s, the enzymatic mixture was incubated for various time intervals (5–40 min). At the end of each incubation period, the absorbance was balanced at 410 nm and 0.05 mL substrate was placed into both cuvettes. The mixture was shaken for 20 s, and the increase in absorbance was recorded for 3 min. In a control experiment, Hepes buffer (0.05 mL) was substituted for the inhibitor solution to establish 100% enzyme activity. The $t_{1/2}$ was determined from the slope of the plot of reciprocal velocity versus time. The association rate constant, k_{on}, was calculated using $t_{1/2}$ and the initial enzyme concentrations.[26,27]

Determination of Dissociation Rate Constant, k_{off}

Buffer, enzyme and inhibitor concentrations were the same as in the preincubation method. Substrate concentration was used in 200-fold molar excess to the inhibitor.

In a typical experiment, the inhibitor (0.05 mL) was added to 2.9 mL Hepes buffer in two quartz cuvettes and thermally equilibrated in the spectrophotometer for 2 min. The cuvettes were shaken for 20 s, and the absorbance was balanced at 410 nm. Enzyme solution (0.005 mL) was placed in the sample cuvette and 0.005 mL acetate buffer was placed in the reference cuvette. The mixture was shaken for 20 s, and was incubated for the time needed for complete inhibition of the enzyme. At the end of the incubation period, the absorbance was balanced at 410 nm and substrate (0.05 mL) was placed into both cuvettes. Both cuvettes were shaken for 20 s, and the increase in absorbance was recorded for 24 h until complete recovery of the enzymatic activity was achieved. Dissociation of the enzyme-inhibitor complex was measured by recording the substrate hydrolysis as a function of time. Dissociation rate constant, k_{off}, was determined from the slope of semilogarithmic plot of the velocity versus time.[28]

RESULTS AND DISCUSSION

The development of a macromolecular inhibitor of HLE is thought to be one of the approaches for therapeutic intervention of proteinase-antiproteinase imbalance states, such as pulmonary emphysema,[3] rheumatoid arthritis[1] and adult respiratory distress syndrome (ARDS).[2] Two factors have been considered in the design of peptidyl carbamates (PC) as elastase inhibitors: (a) specificity of the inhibitor for the enzyme, and (b) reactivity of the carbamate moiety of the inhibitor. The first factor would lead to an optimum "fit" and the formation of a strong Michaelis-Menten type complex between the enzyme and the inhibitor, whereas the second would lead to a faster acylation of the serine residue on the active site of the enzyme. These peptidyl carbamates incorporate a polypeptide consisting of three amino acids and an isostere of an amino acid at the primary site of interaction (S1-P1),[12] thus satisfying the specificity requirement (Figure 1). In order to satisfy the reactivity factor, a good leaving group such as *p*-nitrophenol was chosen at the P′1 region of the inhibitor (Figure 1). Several derivatives of **1** were found to inactivate PPE and HLE selectively *in vitro* and did not affect other serine proteases.[12,13,29] However, their *in vivo* performance has been offset by short biological half-lives ($t_{1/2}$ = 4.0 min) when administered in the hamster lung, which can be attributable to either a rapid elimination from the target compartment or to metabolic degradation.[15]

In the present work we synthesized macromolecular analogs of **1** by linking this peptidyl carbamate inhibitor **1**, to a hydrophilic polymer PHEA **2**. We have utilized Rypacek *et al.*'s approach[14] of incorporation of the spacer ethylene diamine into the polymer which has been shown to produce polymeric analogs of **1** with a sufficient degree of loading and inhibitor flexibility.

The covalent bonding of inhibitor **1** to a polymer PHEA **2** introduces two additional factors involving a Michaelis-Menten type complex between the enzyme and polymeric carbamates; (a) the molecular size of the inhibitor and (b) the extent of loading (number of peptidyl carbamate units per mole of polymer). The first factor could influence the rate of the formation or dissociation of the complex, whereas the second factor could affect the inhibitory potency of the compound due to the multiple inhibitory sites. In order to separate these factors, several macromolecular forms of the peptidyl carbamate inhibitor **1** were synthesized varying the degree of loading from 2 to 6 mole percent (Table I) while using derivatives of PHEA **2** with molecular weight of the polymers ranging from 7 to 27 KDa (Table I).

In order to determine the equilibrium constant K_i, the unbound peptidyl carbamate inhibitor **1** and its polymeric forms, **3** (Scheme 1) were tested under steady state conditions and generated Lineweaver-Burk and Dixon plots. However, a nonlinear replot of reciprocal velocity versus the inhibitor concentration clearly demonstrated the inadequacy of this classic method, which is useful only for

TABLE I HLE inhibition by polymer-bound PC

Compound	M_w^a (KDa)	*Loading*[b] (mol%)	*Incub Time* (min)	IC_{50}^c (μM)	K_i^d (nM)	k_{on}^e $\times 10^3$ $M^{-1}s^{-1}$	k_{off}^f $\times 10^{-6}$(s)
PC1			30	0.46	137	5.4	76
PPC (1)[g]	7	2.0	40	0.29	50	3.2	3.6
PPC (2)	7	3.0	15	0.23	97	4.2	6.5
PPC (3)	7	4.1	15	0.02	35	3.7	1.7
PPC (4)	7	6.3	20	0.14	36	16	0.6
PPC (5)	17	2.0	30	0.25	84	3.6	6.0
PPC (6)	17	4.8	20	0.015	37	10	4.5
PPC (7)	27	2.0	30	0.21	86	3.9	4.6
PPC (8)	27	4.0	20	0.084	23	6.3	2.3
PPC (9)	27	5.5	15	0.031	13	10	1.6

[a] M_w – weight average molecular weight; [b] Determined spectrophotometrically using molar absorptivity of PC; [c] Concentration for 50% remaining activity; [d] n=3 (C.V < 5%); [e] n=3 (C.V. < 6%); [f] n=3 (C.V. < 6%); [g] number in parenthesis denotes experiment number.

inhibitors that reach equilibrium or steady state rapidly. Further examination of the time-dependent loss of the enzymatic activity (Figure 2) showed that the enzymatic activity decreases with the prolonged incubation time, therefore the classic steady state plots did not provide the velocities. Since the Dixon and Lineweaver-Burk analytical methods could not be applied for our series of peptidyl carbamate inhibitor **1** and its polymeric forms **3**, an alternative method of Bieth[25] was utilized. This method can be used provided that the enzyme-inhibitor association is truly reversible and a prolonged incubation period is required to allow complete reaction between HLE and the inhibitor. The inhibition curve, which is the plot of residual enzyme activity versus inhibitor concentration, should be convex. This may occur if 5 minutes incubation time is not sufficient to achieve complete association for the enzyme and the inhibitor.[25] In order to obtain the inhibition curves, the time-dependent HLE inhibition experiments were conducted. These experiments were carried out using the lowest inhibitor concentration. The enzyme was incubated with the inhibitor until residual activity showed no further change with time. Enzyme activity was assayed at different time intervals for different inhibitors after adding the substrate. An adequate incubation period was chosen by taking the shortest incubation period at which the percent remaining activity reached a stable level. The incubation times obtained from these experiments varied from 15–40 minutes (Table I). The result of the time-dependent experiments indicated that increased loading (above 2% of loaded inhibitor) decreases the incubation

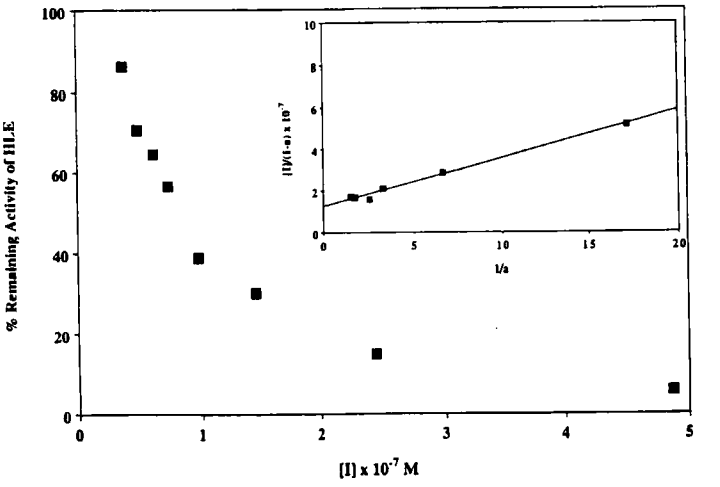


FIGURE 2 Concentration-dependent inhibition of the HLE by PPC (8). The experimental conditions are described in the text. The inset shows an Easson-Stedman plot of the same data. The K_i value for PPC (8) was obtained from the Easson-Stedman equation $[I]/(1-a) = K_i/a + [E^\circ]$.

time with the exception of (polymer-bound peptidyl carbamate, PPC (1), (Table I) which required somehow longer incubation period (40 min) in comparison to free PC (30 min).

Using the results of the time-dependent inhibition experiments, the inhibition curves (percentage remaining activity versus inhibitor concentrations) for all tested inhibitors were constructed (Figure 2). The IC_{50} values were determined from the inhibition curves by finding the inhibitor concentration that produces a velocity that is half of that found in the absence of the inhibitor.

The data obtained from the experiments were also used to construct the Easson-Stedman plots.[26] The slope of these plots was obtained to determine the K_i value for each inhibitor (Figure 2, Table I).

As shown by the K_i values, (Table I), all polymer-bound inhibitors exhibited higher inhibitory activity against HLE compared to low molecular weight (unbound) inhibitor PC **1**. The inhibitory activity of low molecular inhibitor weight PC **1** improved by one order of magnitude after coupling with polymer PHEA **2**. The degree of inhibition was found to be dependent on the degree of loading (number of PC units per mole of polymer). The effect of loading on the inhibition curve is shown in Figure 3, as loading increases IC_{50} and K_i values decreases (Table I). Compare PPC (1)–PPC (4), PPC (5) and PPC (6), and PPC (7)–PPC (9), respectively (Table I). The higher loading affected the concavity of the inhibition curve (Figure 3) and

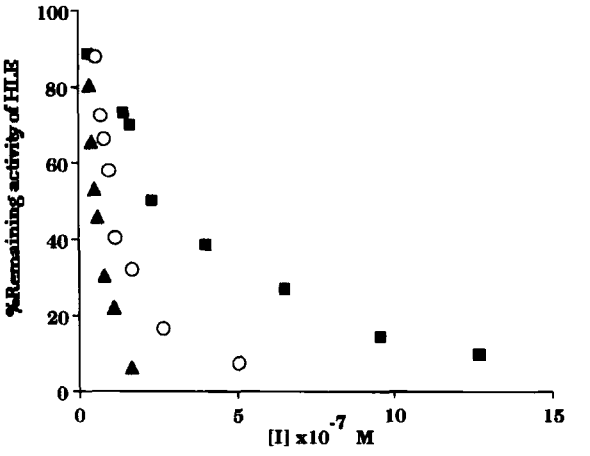


FIGURE 3 The effect of loading on inhibitory activity of polymer-bound PC. Concentration-dependent of HLE by polymer-bound inhibitor: PPC(7) ■, degree of loading 2%, molecular weight of polymer 27,000; PPC1(8) ○, degree of loading 4%, molecular weight of polymer 27,000; and PPC1(5) ▲, degree of loading 5.5%, molecular weight of polymer 27,000. Same conditions as in Figure 2. Enzyme assays were performed in triplicate.

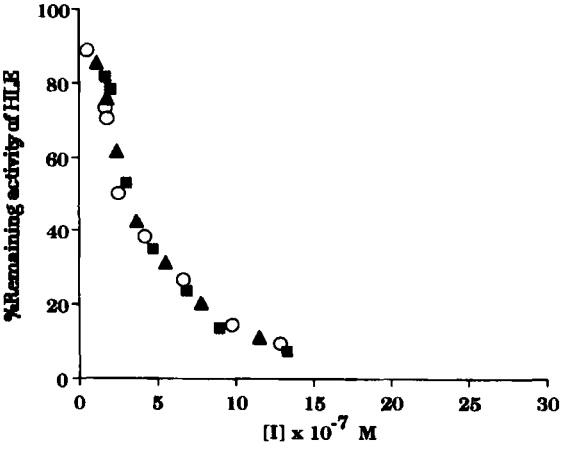


FIGURE 4 The effect of molecular weight on inhibitory activity of polymer-bound PC. Concentration-dependent inhibition of HLE by polymer-bound inhibitor: PPC(7) ○, degree of loading 2%, molecular weight of polymer 27,000; PPC(5) ▲, degree of loading 2%, molecular weight of polymer 17,000; PPC(1) ■, degree of loading 2%, molecular weight 7,000. Same conditions as in Figure 2. Enzyme assays were performed in triplicate.

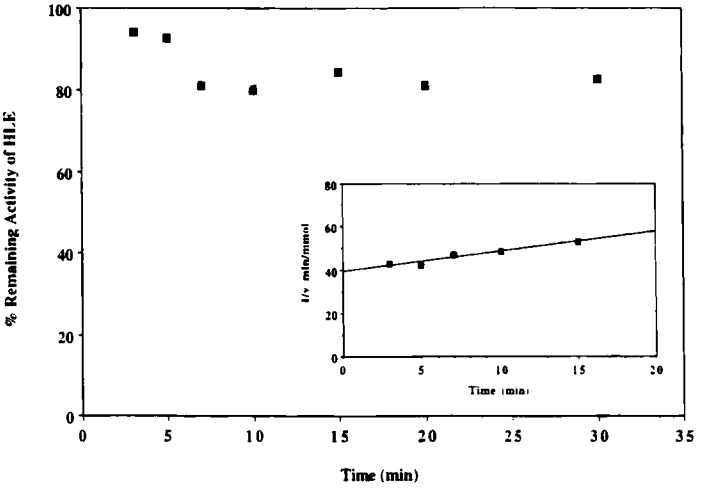


FIGURE 5 Association kinetics of the enzyme and inhibitor PPC(8) complex monitored as % of remaining activity versus time. The experimental conditions are described in the text. The inset shows a graph of the same data which was used to determine the half-life of association[29,30], which was used to calculate K_{on} using the formula $K_{on} = ([E^{\circ}]) \times t_{1/2})^{-1}$.

resulted in an increase in inhibitory capacity, (K_i), Table I. In contrast, the molecular size of the polymer had no effect on the shape of inhibition curve (Figure 4) as well as on the IC_{50} and K_i values of the compounds; compare PPC (1), PPC (5) and PPC (7) in Table I.

Association rate constants, k_{on}, were found to be also dependent on the extent of loading (compounds PPC (1)–PPC (4), PPC (5) and PPC (6), PPC (7)–PPC (9), Table I). Increasing the percentage of loading of the inhibitor on the polymer appears to accelerate the formation of the inhibitor enzyme (EI) complex which seems to be in good agreement with the observed shorter incubation time needed to completely inhibit the enzyme. Surprisingly, an increase in the molecular size of the polymer from 7 to 27 kDa did not effect the association rate constants k_{on} (PPC (1), PPC (5) and PPC (7), Table I, Figure 5). Thus, it was concluded that the increase in molecular weight from 7 to 27 kDa was not significant enough to hinder the inhibitor moiety from inhibitor-enzyme interaction.

Dissociation rate constants, k_{off}, were determined under pseudo-first order conditions (Figure 6) and indicated that inhibition of HLE by low molecular weight PC **1** and its polymeric analogs **3** is a slow reversible process, and k_{off} is affected by the extent of loading as well as the molecular size of polymer. The polymer-bound inhibitors PPC exhibited slower dissociation from enzyme inhibitor complex than

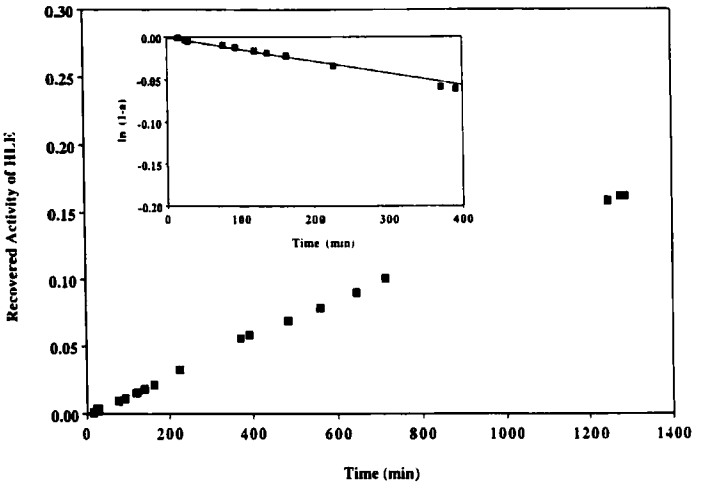


FIGURE 6 Dissociation kinetics (k_{off}) of the enzyme/inhibitor complex determined using a 200 fold excess of substrate. The experimental conditions are described in the text assessed inhibitor is PPC(8). The inset shows a pseudo-first-order representation of the same data.[31]

free inhibitor PC **1** (Table I). The increased loading resulted in slower dissociation of enzyme-inhibitor complex and thus smaller k_{off} as shown in Table I (compounds PPC (1)–PPC (4), PPC (5) and PPC (6), PPC (7)–PPC (9). This finding can be explained by a cooperative interaction of the polymer carrier with the enzyme molecule which might decrease the rate of dissociation.

Stability studies of low molecular weight PC at different pH (6.3, 7.8 and 8.8) have demonstrated the susceptibility of the carbamate bond to hydrolysis. However, the polymer analogs (PPC) of the peptidyl carbamate exhibited significantly slower hydrolysis (Figure 7). A possible explanation for the increased stability of PC coupled with polymer could be due to some engulfment of peptidyl carbamate in the polymer coil, which decreases the probability of nucleophilic attack of the hydroxide ion on the carbamate functionality of PC.

CONCLUSION

Synthesis of several polymeric analogs of low-molecular weight peptidyl carbamate inhibitor and the evaluation of their *in vitro* inhibitory activity against HLE has shown that coupling of PC with a hydrophilic polymer PHEA did not reduce the inhibitory activity or specificity toward the enzyme. In contrast, all polymer bound

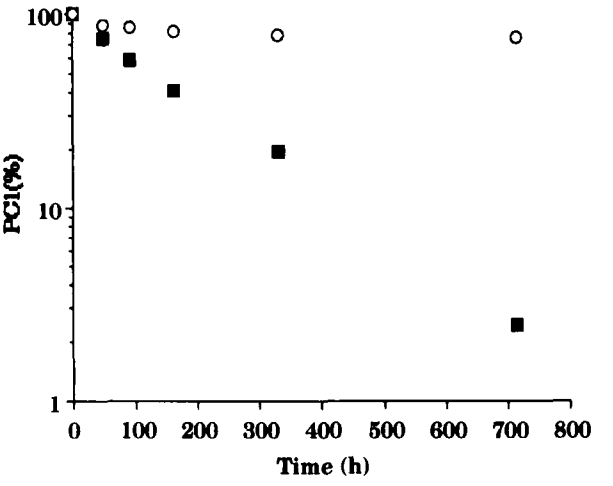


FIGURE 7 Peptidyl carbamates decomposition profile in 0.01 M phosphate buffer pH 8.8, at 37°C ($n = 3$). Polymer-bound inhibitor PPC(○), and free inhibitor PC (■). Initial concentration of PC was 0.048 mg/mL, and initial concentration of PPC was 0.251 mg/mL. The amount contains 0.048 mg of PC/mL. Results represent intact PC as a % of initial PC concentration, in semilogaritmmic plot.

inhibitors exhibited very high potency compared to free PC. Molecular size of the polymer did not effect the inhibitory activity while the degree of loading was found to be an important factor. Enhanced inhibitory activity with increased loading seems to result from the accelerated E-1 complex formation (higher association constant K_{on}).

This study suggests that synthetic macromolecular inhibitors of HLE may provide a useful approach to therapeutic intervention of certain diseases related to an imbalance between HLE and its natural inhibitor, α1-antitrypsin.

References

[1] Ekerot, L. and Ohlsson, K. (1984). *Adv. Exp. Med. Biol.*, **167**, 335–344.
[2] Merritt, T.A., Cochrane, C.G., Holcomb, K., Bohl, B., Hallman, M., Strayer, D., Edwards, D. and Gluck, L. (1983). *J. Clin. Invest.*, **72**, 656–666.
[3] Janoff, A. (1985). *Am. Rev. Respir. Dis.*, **132**, 417–433.
[4] Travis, J. and Salvesen, G.S. (1983). *Annu. Rev. Biochem.*, 52–655.
[5] Powers, J.C., Gupton, B.F., Harley, A.D. Nishino, N. and Whitley, R. (1977). *Biochim. Biophys. Acta*, **485**, 156–166.
[6] Dutta, A.S., Giles, M.B., Gormley, J.J., Williams, J.C. and Kusner, E.J. (1987). *J. Chem. Soc., Perkin Trans.*, **1**, 111–120.
[7] Hassal, C.H., Johnson, W.H., Kennedy, A.J. and Roberts, N.A. (1985). *FEBS Lett.*, **183**, 201–204.
[8] Roberts, N.A. and Surgenor, A.E. (1986). *Biochem. Biophys. Res. Commun.*, **139**, 896–902.

[9] Groutas, W.C., Stanga, M.A., Castrisos, J.C. and Schatz, E.J. (1989). *J. Enz. Inhib.*, **3**, 237–243.
[10] Digenis, G.A., Agha, B.J., Tsuji, K., Kato, M. and Shinogi, M. (1986). *J. Med. Chem.*, **29**, 1468–1476.
[11] Tsuji, K., Agha, B.J., Shinogi, M. and Digenis, G.A. (1984). *Biochem. Biophys. Res. Commun.*, **122**, 571–576.
[12] Kato, M., Agha, B.J., Abdul-Raheem, A., Tsuji, K., Banks, W.R. and Digenis, G.A. (1993). *J. Enz. Inhib.*, **7**, 105–130.
[13] Digenis, G.A., Banks, W.R., Kato, M., Rancher, W.F. and Agha, B.J. (1993). *J. Enz. Inhib.*, **7**, 275–300.
[14] Rypacek, F., Banks, W.R., Noskova, D. and Digenis, G.A. (1994). *J. Med. Chem.*, **37**, 1850–1856.
[15] Noskova, D., Mohammadi, F., Savidge, S.J. and Digenis, G.A. (1993). *J. Enz. Inhib.*, **7**, 303–309.
[16] Banks, W.R., Rypacek, F. and Digenis, G.A. (1991). *J. Labeled Compd. Radiopharm.*, **29**, 381–391.
[17] Stone, P.J., Lucey, E.C., Noskova, D., Digenis, G.A. and Snider, G.L. (1992). *Am. Rev. Respir. Dis.*, **146**, 457–461.
[18] Neri, P., Antoni, G., Benvenuti, F., Cocola, F. and Gazzei, G. (1973). *J. Med. Chem.*, **16**, 893–897.
[19] Vlasak, J., Rypacek, F., Drobnik, J. and Saudek, V. (1979). *J. Polymer Syn.*, **66**, 59–64.
[20] Brown, H.H. (1968). *J. Clin. Chem.*, **14**, 967–978.
[21] Yau, W.W., Kirkland, J.J. and Bly, D.D. (1979). In *Modern Size-Exclusion Chromatography*, John Wiley; New York.
[22] Nakajima, K., Powers J.C., Ashe, B.M. and Zimmerman, M. (1979). *J. Biol. Chem.*, **254**, 4027–4032.
[23] Dixon, M. (1953). *Biochem. J.*, **55**, 170–171.
[24] Lineweaver, H. and Burk, D. (1934). *J. Amer. Chem. Soc.*, **56**, 658–666.
[25] Bieth, J.G. (1980). *Bull. Europ. Physiopath. Resp.*, **16**, (Suppl), 183–195.
[26] Easson, L.H. and Stedman, E. (1936). *Proc. Roy. Soc.*, **B121**, 141–164.
[27] Beatty, K., Bieth, J. and Travis, J. (1980). *J. Biol. Chem.*, **255**, 3931–3934.
[28] Dunlap, R.P., Stone, P.J. and Abeles, R.H. (1987). *Biochem. Biophys. Res. Commun.*, **145**(1), 509–513.
[29] Shinogi, M., Agha, B.J., Tsuji, K. and Digenis, G.A. (1985). *J. Pharm. Sci.*, **74**, 482–485.