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MECHANISTIC STUDY OF HLE INHIBITION USING DUAL LABELED MACROMOLECULAR INHIBITOR[1]

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The mechanism of inhibition of a specific and effective (K_i = 1–10 nM) macromolecular inhibitor of HLE was investigated. The inhibitor, polymer-bound peptidyl carbamate **1** was labeled with [^{3}H] at its polymeric backbone (Mw = 27,000) and with [^{14}C] in it peptidyl carbamate moiety. When the macromolecular inhibitor **1** was incubated with HLE to complete inhibition and then competitively displaced by an HLE substrate, only intact [^{3}H/^{14}C] polymer-bound inhibitor **1** was recovered. At the same time complete restoration of enzymatic activity was achieved. Gel permeation chromatography and HPLC were utilized to eliminate the possibility of the presence of low molecular weight fragments resulting from the interaction of **1** with HLE. It is concluded that **1** exerts its inhibitory action on HLE without the prior release of the low molecular weight inhibitor **3** (Mw = 570).

KEY WORDS: HLE, inhibitors, mechanism

INTRODUCTION

The peptidyl carbamate, *p*-nitrophenyl N-(succinyl-L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropyl carbamate **3** (Figure 1) has recently been found *in vitro* to be a specific and effective inhibitor of HLE[1]. This enzyme is believed to be involved in a number of chronic diseases such as emphysema and arthritis[2]. More recently the ability of **3** to inhibit the elastolytic activity of HLE *in vivo* was investigated[3]. It was found that functionally active **3** disappeared rapidly (4 min for 50% clearance) from the lavageable compartment of the lungs of hamsters after intratracheal instillation. Administration[3] of 100 μg, 500 μg or 3,000 μg of **3** (a 16-, 83- and 500- fold molar excess of the inhibitor) 1 h before instillation of 300 μg of HLE suppressed HLE-induced lung

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Abbreviations: HLE, Human Leukocyte elastase; PPE, Porcine Pancreatic elastase; *p*NA, *p*-nitroanilide; *p*NP, *p*-nitrophenol; MeO-, methoxy; -Suc-, succinyl; GPC, gel permeation chromatography; Me_2SO, dimethyl sulfoxide

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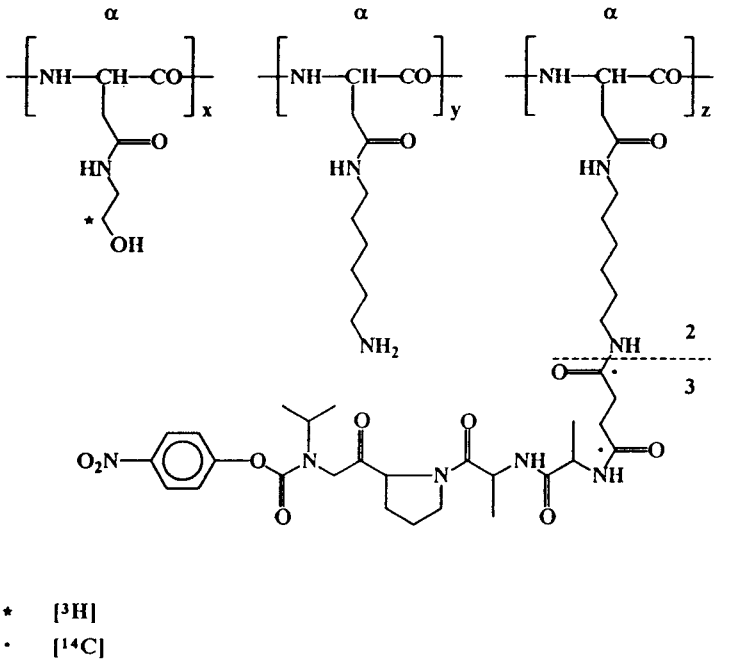

* [^{3}H]
· [^{14}C]

FIGURE 1 Chemical structure of polymer-bound peptidyl carbamate **1** composed of polymeric backbone **2** and peptidyl carbamate moiety **3**. Only α units are shown; x, amount of hydroxy side chains; y, amount of amino side chains; z, amount of peptidyl carbamate side chains.

hemorrhage, but did not moderate HLE-induced emphysema despite the larger molar excess of inhibitor **3**. In contrast, when a 900 μg of the polymer-bound inhibitor **1** (Figure 1), 8.37 molar ratio of **1** to HLE, was administered 1 h before instillation of 300 μg of HLE, a significant amelioration of bronchial secretory cell metaplasia and an almost complete protection from the HLE-induced emphysema in the hamster was observed. Interestingly, the half-time for clearance of **1** from the hamster was found[3] to be 421 min. These results suggested that a rapidly clearing reversible elastase inhibitor **3**, which does not effectively moderate HLE-induced emphysema in the hamster, can be combined with a slowly clearing hydrophilic polymer **2** to produce an effective inhibitor of HLE. The latter may bring about its inhibitory effects without the prior release of the small molecular weight inhibitor from the polymer. The present study was undertaken to probe further the mechanism of action of the macromolecular inhibitor **1** against HLE.

MATERIALS AND METHODS

Materials. Poly-α,β-[N(2-hydroxy-1-[^{3}H]-ethyl)-D,L-aspartamide], **2** (Mw = 27,000; specific activity 0.182 μCi/mg, 43 μCi/mmol), *p*-nitrophenyl-N-([1,4-^{14}C]-succinyl-

L-alanyl-L-alanyl-L-prolylmethyl)-N-isopropyl carbamate, **3** (specific activity 0.679 μCi/mg, 395 μCi/mmol) and [^{3}H/^{14}C] **1** (specific activities [^{3}H] 0.212 μCi/mg, 57 μCi/mmol and [^{14}C] 0.091 μCi/mg, 425 μCi/mmol, the [^{3}H]/[^{14}C] ratio 2.33) were synthesized according previously published procedure[4].

HLE was purchased from Elastin Products Company (Owensville, Missouri). Substrate and Hepes were obtained from Sigma Chemical Company (St. Louis, MO).

Enzyme assay. All enzyme assays were performed spectrophotometrically at 25°C using a Cary Varian 2200 spectrophotometer (Walnut Creek, CA). The activity of HLE was assayed by monitoring the release of *p*-nitroaniline from the substrate MeO-Suc-Ala-Ala-Pro-Val-pNA (λ = 410 μm)[5]. Substrate was dissolved in Me_2SO, inhibitors were dissolved in 0.1 M Hepes buffer, pH 7.5, containing 0.05 M NaCl and 10% (v/v) Me_2SO. HLE was solubilized in 0.5 M sodium acetate buffer, pH 5.5.

Dissociation of enzyme-inhibitor complex. In a typical experiment, 0.15 mL of the inhibitor (2.14 mM) was added to 2.7 mL Hepes buffer, pH 7.5 in the sample cuvette, and thermally equilibrated at 25°C in the spectrophotometer. Subsequently, 0.015 mL of the enzyme (0.49 mM) was added to the sample cuvette and acetate buffer, pH 5.5 (0.015 mL) was added to the reference cuvette. The enzymatic mixture was shaken for 20 s and then incubated at 25°C for 40 min. The absorbance was balanced at 410 nm and 0.14 mL of substrate (136.6 mM)[5] was added to both sample and reference cuvettes. The mixture was shaken for 20 s, and the increase in absorbance at 410 nm was recorded for 24 h until complete restoration of the enzymatic activity was achieved. In a control experiment, Hepes buffer, pH 7.5 (0.15 mL) was used instead of the inhibitor. The level of the enzymatic activity then obtained was considered as 100% activity of HLE[6].

Association of enzyme and inhibitor. The experiment was carried out as described above without addition of the substrate. At the end of the 40 min incubation period the enzyme was completely inactivated as described above. Subsequently, the enzymatic mixture was subjected to ultrafiltration (see HPLC assay).

GPC assay. Separation and fractionation of the enzymatic mixture was performed on a Sephadex G-25 SF column (Pharmacia, Upsala, Sweden) 11 × 140 mm, employing a Waters 650 Advanced Protein Purification System (Millipore, Milford, MA) interfaced with a Waters 481 LC spectrophotometer and a Waters 740 Data Module. A solution containing 0.01 M phosphate buffer, pH 8.8 and 0.15 M NaCl was used as an eluate was monitored spectrophotometrically at 276 nm. One mL fractions were collected at a flow rate of 0.1 mL/min. The individual fractions were mixed with a scintillation cocktail (Scinti Verse II, Fisher Scientific, Cincinnati, OH) in a ratio of 1:3 and subsequently were counted in a liquid scintillation analyzer (Packard 1900 TR, Downers Grove, IL) for [^{3}H] and [^{14}C] activity. The elution curves, [^{3}H], [^{14}C] activity versus elution volume, for **1**, **2** and **3** were generated from [^{3}H], [^{14}C] data. The column was calibrated with authentic samples **1**, **2** and **3**, R_t = 50 min, 50 min and 90 min, respectively.

HPLC assay. HPLC assay was performed by reverse phase HPLC column (Whatman Particil C-8 10 μm, 4.6 × 250 mm) using an Applied Biosystem (Foster City, CA)

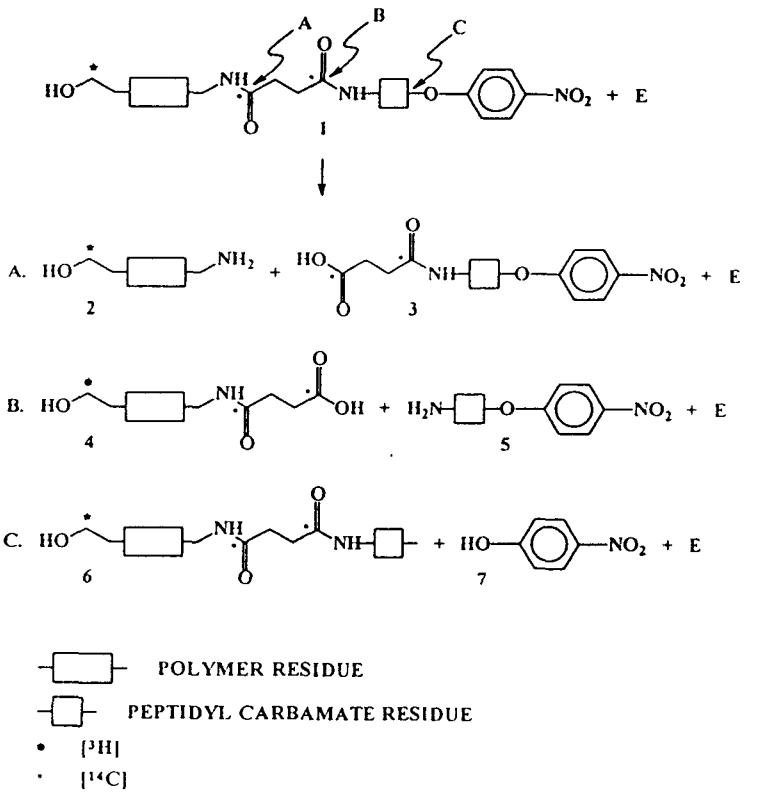

FIGURE 2 Possible pathways of enzymatic attack.

consisting of a 400 solvent Delivery System, an 878 Autosampler, a 787 Programmable Absorbance Detector and an 429 A Integrator. The mobile phase[7] consisted of 1:1 (v/v) of MeOH and 0.1% (w/v) acetic acid, pH 3.1. The enzymatic mixture containing the inhibitor and the completely inactivated HLE in Hepes buffer was subjected to an ultrafiltration procedure (at 1150 rpm) for 25 min, with the use of Amicon micropartition system (Danvers, MA) with an ultrafiltration membrane (YMT, Mw cut off = 1,000). Subsequently, a 100 μL aliquot of the filtrate was injected onto the HPLC column and eluded at a flow rate of 1 mL/min. The uv detector was set up at 275 nm for the detection of **3**, **5** and 315 nm for the detection of **7**. Calibration curves were generated using authentic samples of **5**, **7** and **3**, R_t = 6.0 min, 8.6 min and 13.6 min, respectively.

RESULTS AND DISCUSSION

Possible pathways of the enzymatic attack during the inactivation of HLE by polymer-bound inhibitor **1** are depicted in Figure 2. The most probable attack is at the carbonyl

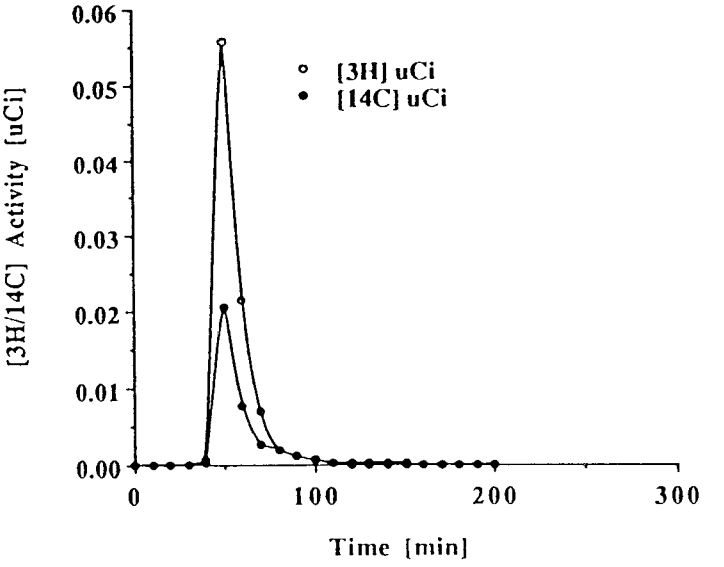

FIGURE 3 [^{3}H]/[^{14}C] elution curve of the enzymatic mixture after complete restoration of the HLE enzymatic activity. **1** is eluted at 50 min. GPC conditions as described in the text. Amounts injected onto the column were: HLE (0.0128 μmol), **1** (0.107 μmol), and substrate (6.364 μmol). Data points were obtained from individual fractions counting. Each data point represents a single experiment performed with triplicate assays.

function of the carbamate ester (at site C) followed by concomitant release of *p*NP **7**. However, other attacks are also possible. Cleavage at site A would result in release of [^{14}C] **3** while cleavage at site B would yield peptidyl carbamate without [^{14}C] terminal hemisuccinate moiety **5**. In order to examine the enzymatic mixture under the conditions of complete inhibition and complete restoration of enzymatic activity, we combined enzymatic assay with HPLC and GPC techniques. HLE, incubated for 40 min with the dual labeled [^{3}H]/[^{14}C] polymer-bound inhibitor **1** (a 45-, 22- and 8- fold molar excess of the inhibitor) in Hepes buffer, was found to be completely inactivated. Subsequently, an excess quantity of the substrate (MeOSuc-Ala-Ala-Pro-Val-*p*NA)[6] solution in Me_2SO was added to the above described enzymatic mixture and incubated for 24 h to displace the inhibitor in the enzyme-inhibitor complex ($k_{off} = 1.63 \times 10^{-5}$ s^{-1}). The complete restoration of the HLE enzymatic activity was monitored by the production of *p*-nitroaniline spectrophotometrically (410 nm)[5]. When a 1.0 mL aliquot sample of the above enzymatic mixture was analyzed by GPC (Sephadex G-25 SF), using a phosphate buffer at pH 8.8, the [^{3}H] and [^{14}C] elution curves showed the presence of intact [^{3}H]/[^{14}C] polymer-bound inhibitor **1**. Figure 3 shows the elution curve of the enzymatic mixture containing HLE (0.0128 μmol), inhibitor **1** (0.107 μmol) and substrate (6.364 μmol). The only peak found in the GPC eluate was [^{3}H]/[^{14}C] polymer peak at 50 min of elution time. No low molecular weight [^{14}C] inhibitor **3** was detected in the GPC eluate.

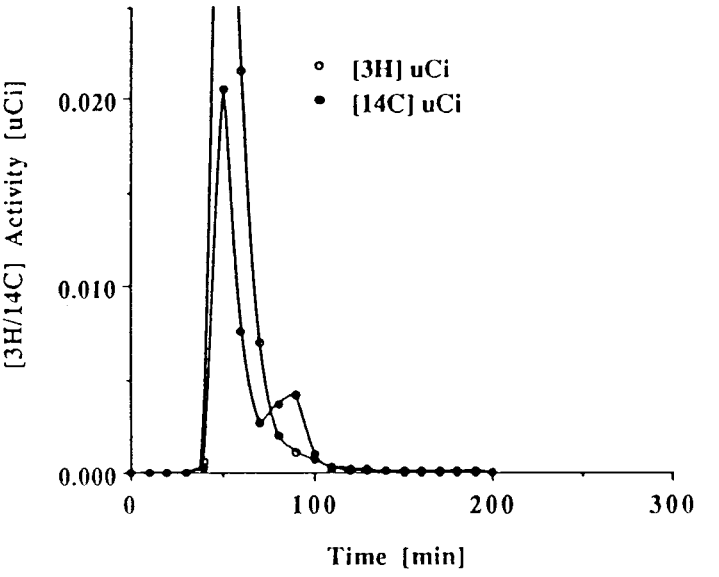

FIGURE 4 [^{3}H]/[^{14}C] elution curve of the enzymatic mixture spiked with [^{14}C] **3**, an equimolar amount to HLE. **1** is eluted at 50 min and **3** is eluded at 90 min. GPC conditions as described in the text. Amounts injected onto the column were: HLE (0.0128 μmol), **1** (0.107 μmol), substrate (6.364 μmol) and **3** (0.0128 μmol). Data points were obtained from individual fractions counting. Each data point represents a single experiment performed with triplicate assays.

We have shown previously[8,9,10] that an excess amount of the low molecular weight inhibitor **3**, or its methyl ester is required to inactivate the enzyme (HLE or PPE). When equimolar amounts of inhibitor **3** and enzyme were incubated for 30 min, only 80% of enzymatic activity was lost[8]. If the polymeric inhibitor **1** were to be cleaved by HLE at site A (Figure 2), it follows that after the complete recovery of the enzymatic activity by the addition of the synthetic substrate[6], at least equimolar amount of [^{14}C] **3** to HLE should be generated from **1**. As a control, the enzymatic mixture was spiked with [^{14}C] **3** (0.0128 μmol), an amount equimolar to HLE, and analyzed by GPC (Figure 4). Addition of the low molecular weight [^{14}C] **3** resulted in appearance of a new peak at 90 min in the GPC eluate. [^{14}C] activity associated with the additional peak was 11,000 dpm, 98% of [^{14}C] **3** added to the enzymatic mixture (Figure 4). Since no alteration in [^{14}C] activity elution curve was detected in the reaction mixture containing inhibitor **1**, HLE and substrate (Figure 3), we conclude that cleavage at site A (Figure 2) does not occur during the inhibition of HLE by dual labeled **1**.

In order to examine the possibility of cleavage at sites B or C (Figure 2) the polymer-bound inhibitor **1** (1.07 μmol) was incubated for 40 min with HLE (0.128 μmol). When HLE was completely inactivated, the enzymatic mixture was subjected to ultrafiltration to eliminate HLE and other macromolecular species such as **1, 2, 4** and **6** (Figure 2). The ultrafiltrate was analyzed for low molecular weight fragments **3, 5**

and **7** (Figure 2) using a previously developed HPLC assay[7]. No detectable amounts of the low molecular weight inhibitor **3** or its fragments **5** and **7** were found in the filtrate (assay detection limit 0.03 μg/mL). In contrast, 97-99% of inhibitor **3** was recovered when the same experiment was performed with inhibitor **3** instead of the polymer-bound inhibitor **1**. Another two control experiments were carried out with authentic samples **5** and **7**. Results of these three control experiments showed that compounds **3, 5** and **7** could be quantitatively determined in the enzymatic mixture under the conditions utilized to inhibit HLE by **1**. Since none of the selected species **3**, **5** and **7** was detected in the experiment in which the reaction mixture contained inhibitor **1** and HLE only, we conclude that cleavage at sites B and C (Figure 2) also does not occur during the interaction of HLE with dual labeled **1**.

Results of these *in vitro* experiments suggest that the polymer-bound inhibitor possesses inhibitory activity against HLE without being released from the polymer. This appears to be in good agreement with *in vivo* findings which showed that the polymer-bound inhibitor was eliminated from the lung compartment at a significantly slower rate ($t_{1/2}$ = 7.5 h) compared to the low molecular weight inhibitor which exhibited a $t_{1/2}$ of 4 min in the lung of the hamster[3]. Provided that low molecular weight inhibitor is released from the polymer no protection from HLE-induced emphysema would be observed. Our mechanistic *in vitro* study has shown that polymer-bound inhibitor interacts with HLE as an intact entity.

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