Inactivation of Purified Human α₂-Antiplasmin and Purified Human Cl Inhibitor by Synthetic Fibrinolytic Agents

By Lindsey A. Miles, John P. Burnier, Michael S. Verlander, Murray Goodman, Alice J. Kleiss, and John H. Griffin

3-Hydroxypropyl flufenamide (Flu-HPA) is one of a series of flufenamic acid derivatives that enhances blood clot lysis in vitro. Studies of possible mechanisms of action of Flu-HPA were undertaken. The fibrinolytic activity of Flu-HPA in clot lysis assays was found to be dependent on plasminogen. The influence of Flu-HPA on the ability of purified α₂-antiplasmin to inhibit purified plasmin was studied. Plasmin activity was determined using 125I-fibrin plates or the spectrophotometric tripeptide substrate, Val-Leu-Lys-paranitroanilide. At Flu-HPA concentrations greater than 1 mM, the inhibitory activity of α₂-antiplasmin was abolished in a time-dependent and concentration-dependent manner. The influence of Flu-HPA on the ability of purified Cl inhibitor to inhibit purified plasma kallikrein and β-Factor XIIa was also studied. Cl inhibitor activity was abolished by Flu-HPA at concentrations greater than 2 mM. Notably, Flu-HPA up to 60 mM did not affect the amidolytic activities of plasmin, kallikrein, or β-Factor XIIa. Flu-HPA did not release enzyme activity from preformed complexes of either α₂-antiplasmin and plasmin or Cl inhibitor and kallikrein. A water-soluble derivative of flufenamic acid, N-flufenamyl-glutamic acid, also inactivated α₂-antiplasmin and Cl inhibitor. This inactivation was shown to be reversible. These results indicate that synthetic fibrinolytic compounds such as flufenamic acid derivatives may promote fibrinolysis by directly inactivating α₂-antiplasmin and Cl inhibitor.

Many small synthetic compounds have been shown to enhance plasma clot lysis in vitro. Urea derivatives were the first compounds studied. Subsequently, attempts were made to design other molecules with certain chemical characteristics in order to identify the structural requirements for enhancement of fibrinolysis. The fibrinolytic activities of different N-arylanthranilates, including flufenamic acid [N(3-trifluoromethylphenyl) anthranilate], a nonsteroidal antiinflammatory agent, were also demonstrated. The mechanism of action of these compounds has not been elucidated. They appear to require plasma components, in addition to plasminogen, to induce fibrinolytic activity. There have been several different hypotheses for the mechanism of action of sodium flufenamate and other related synthetic profibrinolytic molecules. It was suggested that synthetic fibrinolytic agents dissociate enzyme-inhibitor complexes such as plasmin-antiplasmin or that they solubilize and remove inhibitors of plasminogen activators from a fibrinolytic enzyme complex. A positive, qualitative correlation between the ability of synthetic fibrinolytic compounds to remove antiplasmin activity and to lyse fibrin clots was made. It has also been suggested that blocking of plasma inhibitors of urokinase is effected by certain compounds and that α₂-macroglobulin, anti-thrombin III, and Cl-inhibitor are inactivated by various synthetic fibrinolytic compounds.

Recently, the occurrence in plasma of a fast inhibitor of plasmin, α₂-antiplasmin has been described. It reacts very rapidly to form a 1:1 complex with plasmin and is the major inhibitor of plasmin in plasma. Prandini et al. demonstrated that two synthetic fibrinolytic agents, orthothymotic acid and α(isobutyl-4-cyclohexene-1-yl) propionic acid (S-1623), inactivate purified α₂-antiplasmin and, at higher concentrations, also inactivate plasmin. It has also been suggested that Cl inhibitor is inactivated by sodium flufenamate although no evidence for a direct effect of flufenamic acid on purified Cl inhibitor has appeared. Cl inhibitor is a plasma protease inhibitor that inhibits C1r, C1s, activated Factor XII, activated Factor XI, and plasma kallikrein. The latter three enzymes have been reported to activate plasminogen. Thus, inactivation of Cl inhibitor by flufenamic acid derivatives could enhance fibrinolysis through inactivation of an inhibitor of several possible plasminogen activators.

The effect of a derivative of flufenamic acid, 3-hydroxypropyl flufenamide (Flu-HPA), on both purified α₂-antiplasmin and purified Cl inhibitor is assessed in this article. Flu-HPA has been synthesized and shown to be more active than sodium flufenamate in clot lysis...
In this study it is shown that Flu-HPA, directly inactivates α₂-antiplasmin and Cl inhibitor but does not inactivate plasmin, plasma kallikrein, or β-Factor XIa. Moreover, such an effect of one water-soluble derivative of flufenamic acid on α₂-antiplasmin and on Cl inhibitor is shown to involve a reversible reaction.

MATERIALS AND METHODS

All chemicals obtained from commercial sources were the best grade available. Plasminogen was prepared by affinity chromatography on lysine Sepharose, precipitation with 50% ammonium sulfate, and chromatography on a column containing DEAE-Sephadex A-50 equilibrated with 40 mM Tris-Cl, pH 8.2, 10 mM Na succinate, 13 mM NaCl, 0.02% Na azide. The column was washed with 50 ml equilibration buffer after sample application. Plasminogen was eluted with a linear salt gradient of 120 ml equilibration buffer in the proximal chamber and 120 ml equilibration buffer with NaCl added to give a conductivity of 20 mmho in the distal chamber. Plasmin was prepared by a modification of the method of Wiman and Wallen. First, plasminogen was dialyzed against buffer containing 0.1 M Na phosphate, pH 7.6, 0.02% Na azide. Glycerol was added to give a 25% v/v ratio. The mixture was added to Affigel 10 (Biorad) beads to which urokinase (Calbiochem) had been covalently coupled. After 4.5 hr of incubation at 30°C, the incubation mixture was centrifuged and the supernatant was removed and centrifuged several times to remove all of the beads. Plasmin amidolytic activity on the tripeptide substrate S-2251 (H-D-Val-Leu-Lys-paranitroanilide) (Kabi, Sweden) was determined and compared with a plasmin sample that had been titrated with the active site titrant parantitrophenyl guanidino benzoate. Aliquots of plasmin were stored in small volumes at −70°C. Human prekallikrein was purified to greater than 95% homogeneity, as judged by SDS-polyacrylamide gels, according to the method of Bouma et al. Kallikrein was purified as described. Human Factor XII was purified to greater than 95% homogeneity using published procedures. β-Factor XIa was prepared as described. α₂-Antiplasmin was purified using a modification of the methods of Moron and Aoki and Wiman and Collen. A quantity of 1.4 liters of citrated normal human plasma was passed over a 2.5 x 71 cm lysine-Sepharose affinity column equilibrated with 0.1 M Na phosphate, pH 7.4, containing 0.02% Na azide. The fractions that did not bind to lysine Sepharose were pooled. The portion of this pool that was soluble in 30% ammonium sulfate and precipitated in the presence of 50% ammonium sulfate was dialyzed against 75 mM Na phosphate, pH 7.6, 0.02% Na azide. The sample was applied to a 5 x 37 cm column containing DEAE-Sephadex A-50 that had been equilibrated with 0.1 M Na phosphate pH 7.6, 0.02% Na azide. The column was washed with 1350 ml equilibration buffer after sample application. A linear salt gradient of 900 ml of equilibration buffer in the proximal chamber and 900 ml of equilibration buffer with NaCl added to give a concentration of 0.2 M in the distal chamber was used to elute α₂-antiplasmin. Fractions containing α₂-antiplasmin were pooled, lyophilized, and dissolved in 10 mM Na phosphate, pH 7.6, 0.02% Na azide. The pooled and made 1 mM with β-mercaptoethanol and applied to a 1.6 x 4 cm concanavalin-A-Sepharose column equilibrated with 40 mM Na phosphate, pH 7.0, 0.02% Na azide. The column was washed with approximately 35 ml of this buffer after sample application. α₂-Antiplasmin was eluted with this buffer containing 20 mM α-methylmannoside. The purified protein had an apparent molecular weight of 70,000, as judged by SDS-polyacrylamide gel electrophoresis, and was at least 90% pure as judged by these gels.

Frations containing α₂-antiplasmin activity were dialyzed individually against 40 mM Na phosphate, pH 7.5, 0.02% Na azide, 1 mM β-mercaptoethanol, and stored at −70°C.

Purification of Cl Inhibitor

Freshly citrated human plasma was the starting material for isolation of Cl inhibitor. Blood was collected from the antecubital vein of normal healthy volunteers into 50-ml plastic syringes and was rapidly mixed in centrifuge tubes with one-sixth volume acid-citrate-dextrose anticoagulant (ACD: 1 liter contains 13.6 g citric acid, 25 g sodium citrate, 20 g dextrose). The blood was centrifuged at 3000 g for 20 min at 20°C and the plasma obtained was centrifuged again at 5000 g for 40 min at 20°C.

All purification steps were carried out using plasticware or siliconized glassware (Siliclad, Clay Adams) at 4°C except for the initial dialysis of plasma and the first DEAE-Sephadex column, which was performed at 20°C. All buffers contained 1 mM benzamidine HCl, 1 mM Na azide, 50 µg/ml polybrene (Aldrich), and 0.02% Na azide. Buffers were prepared fresh for each step. Dialysis tubing and all containers were prerinsed with 2 g/liter Polybrene solution and then rinsed with H2O before contacting the solution containing Cl inhibitor.

The following is a convenient scheme for isolating Cl inhibitor from 1.4 liters of ACD plasma. Throughout the procedure, Cl inhibitor was detected by double immunodiffusion analysis, or radial immunodiffusion, using rabbit antisera to human Cl inhibitor (Behring Diagnostics, Somerville, N.J.).

Step 1. DEAE-Sephadex chromatography. Plasma was dialyzed at 20°C against the starting buffer (40 mM Tris-Cl, 10 mM succinic acid, pH 8.2, µ – 2–3 mmho) before chromatography on a 10.5 x 42 cm column containing 100 g of DEAE-Sephadex A-50 as described elsewhere. The column was washed with 3600 ml starting buffer after sample application. A linear gradient of 6 liters of starting buffer in the proximal chamber and 6 liters of Tris-Cl, 0.12M succinic acid, 0.3M NaCl, in the distal chamber was used to elute Cl inhibitor from the column. Cl inhibitor was eluted after the albumin peak and before high Mr, kininogen.

Step 2. SP-Sephadex chromatography. The fractions containing Cl inhibitor were pooled, dialyzed, and applied to a SP-Sephadex-C50 column (2.5 x 28 cm) that had been equilibrated with the starting buffer (50 mM Na acetate-acetic acid, pH 5.3). The column was washed with 140 ml of starting buffer before the gradient was applied. Cl inhibitor was eluted using a linear gradient of 350 ml starting buffer in the proximal chamber and 350 ml starting buffer with NaCl added to give a concentration of 0.25M NaCl in the distal chamber. Cl inhibitor eluted between 7 and 15 mmho.

Step 3. DEAE-Sephadex chromatography. The fractions containing Cl inhibitor were pooled, dialyzed, and applied to a 2.5 x 13 column containing DEAE-Sephadex that had been equilibrated with the starting buffer (50 M Na acetate-acetic acid, 70 mM NaCl, pH 5.3). After the sample was applied, the column was washed with 65 ml starting buffer. A linear gradient of 200 ml starting buffer in the proximal chamber and 200 ml of starting buffer containing 0.4 M NaCl, in the distal chamber, was then applied. Cl inhibitor eluted between 16 and 29 mmho.
preparation was approximately 90% pure as determined on SDS gels.

Antibodies to human plasminogen were produced in goats by injection of 2.5 mg of human plasminogen over a 4-wk period. Serum from the fifth week bleed and normal unimmunized goat serum were treated in the following manner to isolate the gamma globulin fraction: Serum was adsorbed 3 times with 10 mg/ml kaolin for 20 min at 37°C, and then heat treated at 56°C for 1 hr. The serum was dialyzed against 10 mM Na phosphate, pH 7.7, and batch absorbed with DEAE cellulose using 10 g of resin/g of protein for 30 min at 22°C. The supernatant was precipitated with 50% ammonium sulfate and dialyzed against 10 mM Tris-Cl, pH 7.5, 0.4 M NaCl, 0.02% Na azide.

Fibrinogen was isolated from human plasma using sequential precipitations. Human thrombin was a gift from Dr. David Aronson of the Bureau of Biological Standards. The preparation used was thrombin prep XXXIV in 0.75 M NaCl, pH 6.0, 10,350 U/ml, prepared by Dr. John Fenton, Albany, N.Y.

The normal human plasma pool consisted of plasma anticoagulated with ACD from 20 normal volunteers.

3-Hydroxypropyl flufenamide, N-flufenamyl-glutamic acid disodium salt, and N-flufenamyl-β-alanine sodium salt were synthesized using standard chemical methods. The chemical structures of these compounds are illustrated in Fig. 1.

Protein concentrations were determined by the Lowry method using bovine serum albumin (Sigma Chemicals) as a reference. pH measurements were performed at room temperature using a Radiometer model 26 pH meter (Radiometer, Copenhagen). Conductivity was measured at room temperature with a Radiometer conductivity meter, type CDM 2d, using a 0.54 cm cell, type CDC 114.

**Assays of α2-Antiplasmin Activity**

α2-Antiplasmin activity was assessed using a fibrin plate assay or a chromogenic tripeptide substrate, S-2251, as previously described. In a typical amidolytic assay of the ability of Flu-HPA to inactivate α2-antiplasmin, 2 μl of various concentrations of Flu-HPA dissolved in distilled DMF or DMF alone, plus 3 μl of buffer (0.5 M Tris-Cl, pH 8.11, 0.15 M NaCl, 0.02% Na azide) were added to a 10 × 75 mm glass tube. Fifteen microliters of α2-antiplasmin, diluted in TBA-BSA, to give a final concentration of 83 nM were added. The reaction mixture was centrifuged to bring all reactants to the bottom of the tube, incubated for 30 min at 37°C, and cooled at 0°C. Fifteen microliters of plasmin diluted in TBS-BSA to give a final concentration of 135 nM, at 4°C, were added and the mixture was incubated at room temperature for 30 sec. Seventy microliters of S-2251 at 0.48 mg/ml in 50 mM Tris-Cl, pH 7.4, 0.02% Na azide, 0.012 M NaCl, at room temperature were added; 100 μl of the mixture were removed from the tube and placed in a narrow black masked cuvette with a 1-cm path length. The change in absorbance per minute at 405 nm was recorded, using a Gilford model 2400 spectrophotometer equipped with a Gilford 6050 Chart recorder. 125I-fibrin plates were prepared using a modification of the method of Unkeless et al. Fibrinogen was labeled using the lactoperoxidase method. A mixture of labeled and unlabeled fibrinogen was diluted with deionized water to a concentration of 56 μg/ml containing 2 × 106 cpm/ml. Fifty microliters of this solution was pipetted into each well of a flat-bottom Costar (M.A. Bioproducts, Los Angeles, Calif.) tissue culture plate that contained 96 wells with 6-mm diameters and capacities of 200 μl. The fibrinogen solution was dried at 37°C and the plates were kept at 4°C until used. Immediately before use, the dried fibrinogen was clotted by addition of 50 μl purified human α-thrombin (116 nM) in 0.1 M Tris-Cl, pH 8.2, 0.02% NaN₃. The plates were incubated at 37°C for 1 hr. The thrombin solution was removed. The wells were then washed 3 times with 100 μl of the above buffer. To assay plasmin fibrinolytic activity, 100-μl samples in triplicate were placed in wells, and 10-μl aliquots were removed at various times and counted in a Micromedic 4/600 gamma counter.

The thrombin used to clot the fibrinogen was prepared from purified human prothrombin™ using activation with Echis carinatus venom followed by Biorex 70 chromatography. Five-hundred milliliters of prothrombin at 0.36 mg/ml in 0.05 M sodium phos-

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*Miles, Rothschild and Griffin, unpublished results.

†Marlar and Griffin, manuscript in preparation.
phate, pH 7.0, and Echis carinatus venom at 20 µg/ml in the same buffer were separately absorbed with Biorex 70. They were then mixed and incubated at 37°C for 1 hr. The mixture was passed over a Biorex 70 column equilibrated with the same buffer. The column was washed with equilibration buffer. Thrombin was eluted from the column with 0.3 M sodium phosphate, pH 8.0.

Assays of Cl Inhibitor Activity

Cl inhibitor activity was assessed by measuring its inhibition of the kallikrein-dependent cleavage of the tripeptide substrate benzoyl-Pro-Phe-Arg-paranitroanilide (Vega Fox, Ariz.) or of the β-Factor-XIIa-dependent cleavage of the tripeptide substrate S-2302 (H-D-Pro-Phe-Arg-paranitroanilide) (Kabi, Sweden).

In a typical assay of the ability of Flu-HPA to inactivate Cl inhibitor, the following protocol was used. Two microliters of Flu-HPA diluted to various concentrations in distilled DMF or DMF alone, 10 µl of TBS-BSA, 3 µl of buffer (0.5 M Tris-Cl, pH 8.15, 0.15 M NaCl, 0.02% Na azide), 5 µl of Cl inhibitor diluted in TBS-BSA to give a final concentration of 367 nM and 10 µl kallikrein, which gave a final concentration of 446 nM, were added to a 10 × 75 mm siliconized glass tube and incubated for 20 min at 37°C. Fifty microliters of TBS-BSA at 4°C and 70 µl of TBS at 4°C were added. One and one-half milliliters of benzoyl-Pro-Phe-Arg-paranitroanilide 0.1 mg/ml (0.14 mM) in 0.1 M Tris-Cl, pH 8.3, 0.05 M NaCl, 0.02% Na azide were added immediately, and the reaction mixture was placed in a cuvette with a path length of 0.5 cm. Amidolytic activity was measured as the initial rate of change in absorbance at 405 nm/min. The initial rate was linear for addition of 2-48.5 nmol of kallikrein to the tripeptide substrate.

In other assays, 2 µl of Flu-HPA diluted to various concentrations in distilled DMF or DMF alone, 10 µl TBS-BSA, 2 µl of buffer (0.5 M Tris-Cl, pH 8.15, 0.15 M NaCl, 0.02% Na azide), and 5 µl of Cl inhibitor that was diluted in TBS-BSA to give a final concentration of 26 nM were added to a 10 × 75 mm siliconized glass tube. The mixture was centrifuged to bring reactants to the tube bottom and incubated for 30 min at 37°C. Five microliters of β-Factor XIIa, diluted in TBS-BSA to 14 µg/ml, giving a final concentration of 36 nM, were added to the tube. The mixture was incubated for an additional 20 min at 37°C. Twenty microliters of the mixture were removed and added to a microcuvette with a 1-cm path length that contained 80 µl of S-2302, 0.125 mg/ml in 0.1 M Tris-Cl, pH 8.0, 0.05 M NaCl, 0.02% Na azide. Amidolytic activity was determined by recording the change in absorbance at 405 nm/min.

Washed clot lysis assays were performed as previously described. Clots were formed by recalcification of citrated human plasma usually obtained from outdated blood-bank plasma. Decalcified platelet-poor plasma (0.1 ml) was added to a solution of 0.15 M sodium chloride (0.1 ml), 0.05 M imidazole (0.1 ml), and 0.025 M calcium chloride (0.1 ml). After approximately 30 min, the clots were removed, washed with 2 ml of 0.15 M sodium chloride, and deposited in a 10 × 75 mm test tube containing 0.4 ml of 0.15 M sodium chloride and the test material. Clots were kept at 37°C and time for lysis was recorded.

RESULTS

Plasminogen Dependence of Clot Lysis Enhancement by Flu-HPA

In order to test whether the clot lysis enhancement by Flu-HPA that had been previously observed was plasminogen dependent, the ability of goat antiplasminogen antibodies to inhibit clot lysis was studied. Incorporation of Flu-HPA into a clot containing normal goat gamma globulin shortened the time required for complete clot lysis from 74 ± 3 hr to 49.5 ± 3 hr. In the presence of antiplasminogen antibodies, clots did not lyse in 143 hr even in the presence of Flu-HPA. This shows that plasminogen is required for the profibrinolytic activity of Flu-HPA.

Effect of Flu-HPA on the Reaction Between Plasmin and α2-Antiplasmin

The influence of Flu-HPA on the activity of α2-antiplasmin was studied. Plasmin activity was determined spectrophotometrically as lysine amidase activity, and α2-antiplasmin activity was measured by its ability to inhibit plasmin activity. A concentration of α2-antiplasmin that did not fully inhibit plasmin activity was chosen so that small effects of Flu-HPA could be detected. The activity of α2-antiplasmin was inhibited 50% by 0.4 mM Flu-HPA and entirely abolished at higher concentrations, as seen in Fig. 2A. At concentrations of Flu-HPA up to the highest concentration tested, 70 mM, this compound had no effect on the activity of plasmin (Fig. 2B). Similar results were obtained when Flu-HPA was dissolved in methanol instead of DMF. Thus, the small amounts of DMF used to dissolve Flu-HPA had no effect on the interaction of the proteins. From these observations it is concluded that Flu-HPA directly inactivates α2-antiplasmin.

The influence of the bovine serum albumin concentration on the reaction between Flu-HPA and α2-antiplasmin in these experiments was investigated. When the TBS-BSA was adjusted to a final concentration of 4.3 mg/ml of bovine serum albumin, instead of 0.43 mg/ml concentration routinely used, there was no effect of albumin on inactivation of α2-antiplasmin by Flu-HPA. However, above this concentration of albumin, inactivation of α2-antiplasmin by Flu-HPA was decreased, and the effect of Flu-HPA (5.14 mM) was eliminated at 21.5 mg/ml albumin (0.35 mM). Thus, the concentrations of bovine albumin used in our experiments did not appear to influence the effects of Flu-HPA and it is noted that high levels of albumin can influence these assays, presumably by binding Flu-HPA.

The time dependence of the reaction between Flu-HPA and α2-antiplasmin was studied. Under the reaction conditions used, α2-antiplasmin was totally inactivated after incubation with 6.7 mM Flu-HPA for 2 min.

Effect of Flu-HPA on the Ability of α2-Antiplasmin to Inhibit the Cleavage of Fibrin by Plasmin

The effect of Flu-HPA on the ability of α2-antiplasmin to inhibit the activity of plasmin on fibrin, its
natural substrate, was tested (Fig. 3). A concentration of α2-antiplasmin that did not fully inhibit plasmin was chosen in order to detect small effects of Flu-HPA. In the absence of Flu-HPA, 60% inhibition was observed. Between 0.5 and 1 mM Flu-HPA, the activity of α2-antiplasmin was abolished. Thus, Flu-HPA inactivates the ability of α2-antiplasmin to inhibit plasmin hydrolysis of fibrin.

Between 0 and 2 mM Flu-HPA there was a slight inhibition of the fibrinolytic activity of plasmin. This effect of 20% inhibition of plasmin by Flu-HPA was very reproducible. In contrast to this phenomenon, Flu-HPA had no inhibitory effect on the amidolytic activity of plasmin.

Effect of Flu-HPA on the Reaction Between Plasma Kallikrein and C1 Inhibitor

The influence of Flu-HPA on the ability of C1 inhibitor to inhibit plasma kallikrein was studied. Kallikrein activity was determined spectrophotometrically as amidase activity, and C1 inhibitor activity was measured by its ability to inhibit kallikrein. A concen-
Fig. 3. Effect of Flu-HPA on the ability of α2-antiplasmin to inhibit the cleavage of fibrin by plasmin. Thirty microliters of various dilutions of Flu-HPA in DMF plus 150 μl of α2-antiplasmin diluted in TBS-BSA to give a final concentration of 0.18 nM were added to a 1 ml polypropylene snap cap tube and incubated at 37°C for 30 min. The tubes were placed on ice for 5 min and then 150 μl of plasmin, which gave a final concentration of 0.30 nM, were added. One-hundred microliters from each sample were placed in each of three fibrin plate wells as described in Materials and Methods. The dashed line and open circles indicate the activity of α2-antiplasmin. The solid line and closed circles indicate the activity of plasmin alone. When 24 mM Flu-HPA was tested (not shown), the results were the same as for 12 mM Flu-HPA.

Fig. 4. Effect of Flu-HPA on the reaction between plasma kallikrein and Cl inhibitor. The reaction conditions are described in Materials and Methods. The dashed line and open circles indicate the activity of Cl inhibitor plus kallikrein. The solid line and closed circles indicate the activity of kallikrein alone.

Fig. 5. Effect of Flu-HPA on the reaction between β-Factor XIIa and Cl Inhibitor

The effect of Flu-HPA on the ability of Cl inhibitor to inhibit β-Factor XIIa was also studied. β-Factor XIIa activity was determined spectrophotometrically as amidase activity, and Cl inhibitor activity was measured by its ability to inhibit β-Factor XIIa. A concentration of Cl inhibitor that did not completely inhibit β-Factor XIIa activity was chosen in order to detect small effects of Flu-HPA on the protein interactions. Figure 5 shows that the activity of Cl inhibitor was totally eliminated at Flu-HPA concentrations greater than 2 mM, while β-Factor XIIa activity was not affected by concentrations of Flu-HPA up to the highest concentration tested, 160 mM. Similar results were obtained when Flu-HPA was dissolved in methanol, rather than DMF. Therefore, the small quantities of DMF used to dissolve Flu-HPA had no effect on the interaction of Cl inhibitor with β-Factor XIIa. These results, like those above, show that Flu-HPA directly inactivates Cl inhibitor.

Effect of Flu-HPA on a Preformed Complex of Plasmin and α2-Antiplasmin

The effect of Flu-HPA on a preformed complex of plasmin and α2-antiplasmin was studied to determine
**Inactivation of α2-Antiplasmin and C1 Inh.**

Table 1. Effect of Flu-HPA on a Preformed Complex of Plasmin and α2-Antiplasmin*

<table>
<thead>
<tr>
<th>Preincubated</th>
<th>Added</th>
<th>Plasmin Activity (ΔA405/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmin + α2-antiplasin</td>
<td>Flu-HPA</td>
<td>0.007</td>
</tr>
<tr>
<td>Plasmin + α2-antiplasin</td>
<td>Buffer</td>
<td>0.005</td>
</tr>
<tr>
<td>Flu-HPA + α2-antiplasin</td>
<td>Plasmin</td>
<td>0.124</td>
</tr>
<tr>
<td>Plasmin + buffer</td>
<td>Flu-HPA</td>
<td>0.096</td>
</tr>
<tr>
<td>Plasmin + buffer</td>
<td>Buffer</td>
<td>0.116</td>
</tr>
</tbody>
</table>

*15 μl of α2-antiplasmin, diluted in TBS-BSA to give a final concentration of 118 nM, 15 μl of plasmin diluted in TBS-BSA to give a final activity of 135 nM, and 3 μl of 0.5 M Tris-Cl, pH 8.1, 0.15 M NaCl, 0.02% Na azide were added to a 10 × 75 mm glass tube and incubated for 5 min at 37°C. 2 μl of Flu-HPA at 87 mM in DMF or DMF alone were added, and the mixture was incubated for an additional 10 min at 37°C. Or α2-antiplasmin, Flu-HPA, and buffer were mixed and incubated at 37°C for 10 min. Then plasmin was added and the mixture was incubated for an additional 5 min at 37°C. Plasmin activity was assayed as amidolytic activity.

Effect of Flu-HPA on a Preformed Complex of Kallikrein and C1 Inhibitor

The effect of Flu-HPA on a preformed complex of plasma kallikrein and Cl inhibitor was studied to determine whether Flu-HPA could dissociate the complex and thus liberate plasmin activity. When α2-antiplasmin was preincubated with plasmin before the addition of Flu-HPA, no plasmin activity was liberated (Table 1). The control shows that preincubation of α2-antiplasmin with Flu-HPA results in no loss of plasmin activity. Thus, Flu-HPA did not release plasmin activity from a preformed complex of plasmin and α2-antiplasmin.

Effect of Flu-HPA on a Preformed Complex of Kallikrein and Cl Inhibitor

The effect of Flu-HPA on a preformed complex of plasma kallikrein and Cl inhibitor was studied to determine whether Flu-HPA could dissociate the complex and thus liberate kallikrein activity from the complex. When Cl inhibitor was preincubated with kallikrein before the addition of Flu-HPA, no kallikrein activity was regenerated (Table 2). In this table, whether Flu-HPA could dissociate the complex and thus liberate plasmin activity. When α2-antiplasmin was preincubated with plasmin before the addition of Flu-HPA, no plasmin activity was liberated (Table 1). The control shows that preincubation of α2-antiplasmin with Flu-HPA results in no loss of plasmin activity. Thus, Flu-HPA did not release plasmin activity from a preformed complex of plasmin and α2-antiplasmin.

Table 2. Effect of Flu-HPA on a Preformed Complex of Kallikrein and C1 Inhibitor*

<table>
<thead>
<tr>
<th>Preincubated</th>
<th>Added</th>
<th>Kallikrein Activity (ΔA405/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl Inhibitor + kallikrein</td>
<td>Flu-HPA</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cl Inhibitor + kallikrein</td>
<td>Buffer</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Flu-HPA + Cl Inhibitor</td>
<td>Kallikrein</td>
<td>0.020</td>
</tr>
<tr>
<td>Buffer</td>
<td>Kallikrein</td>
<td>0.028</td>
</tr>
<tr>
<td>Buffer + Flu-HPA</td>
<td>Kallikrein</td>
<td>0.025</td>
</tr>
</tbody>
</table>

*25 μl of Cl inhibitor diluted in TBS-BSA to give a final concentration of 39 nM, 3 μl of buffer (0.5 M Tris-Cl, pH 8.15, 0.15 M NaCl, 0.02% Na azide), and 5 μl of kallikrein diluted in TBS-BSA to give a final concentration of 40 nM were added in order to a 10 × 75 mm siliconized glass tube and incubated at 37°C for 5 min. 2 μl of 1.14 M Flu-HPA in DMF or DMF alone were added and further incubated for 10 min at 37°C. Or as indicated, Cl inhibitor, Flu-HPA, and Tris buffer were preincubated for 10 min at 37°C, and then kallikrein was added and the mixture further incubated for 5 min at 37°C. Kallikrein activity was assayed as amidolytic activity as described in Materials and Methods.

Table 3. Comparison of the Activities of Water-Soluble Flufenamic Acid Derivatives on the Inactivation of Purified α2-Antiplasmin and on Fibrinolysis in Washed Clot Lysis Assays

<table>
<thead>
<tr>
<th>Derivative</th>
<th>2 mM</th>
<th>10 mM</th>
<th>Lysis of Washed Clot in Less Than 36 hr*</th>
<th>Concentration Required for 50% Inhibition of α2-Antiplasmin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Flu-Glu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6.3 mM</td>
</tr>
<tr>
<td>N-Flu-β-Ala</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.3 mM</td>
</tr>
</tbody>
</table>

*Washed clot lysis assays were performed as described in Materials and Methods. The final concentration of α2-antiplasmin in the washed clot lysis assay is 125 nM, assuming a value of 1 μM for the concentration of α2-antiplasmin in plasma.

†The assay system is the same as that described in Materials and Methods for the effect of Flu-HPA on the reaction between plasmin and α2-antiplasmin. The concentration of α2-antiplasmin was 107 nM and the concentration of plasmin was 134 nM. Flufenamic acid derivatives were dissolved in H2O.

Comparison of the Activities of Water-Soluble Flufenamic Acid Derivatives on the Inactivation of Purified α2-Antiplasmin and on Fibrinolysis in Washed Clot Lysis Assays

The influence of two water-soluble flufenamic acid derivatives on the activity of α2-antiplasmin was studied. The concentrations of these derivatives at which 50% inactivation of α2-antiplasmin was observed is given in Table 3. N-flufenamyl-glutamic acid (N-Flu-Glu) and N-flufenamyl-β-alanine (N-Flu-β-Ala) the appropriate control shows that preincubation of Cl inhibitor with Flu-HPA inactivated Cl inhibitor. Thus, Flu-HPA is not capable of dissociating this enzyme-inhibitor complex to yield free enzyme.

Fig. 6. Effect of sodium flufenamate on the reaction between plasmin and α2-antiplasmin. The assay procedure is the same as that described in Materials and Methods for the effect of Flu-HPA on the reaction between plasmin and α2-antiplasmin. The concentration of plasmin was 134 nM. Sodium flufenamate was dissolved in H2O. The dashed line and open circles show the activity of α2-antiplasmin plus plasmin. The solid line and closed circles indicate the activity of plasmin alone.
did not inactivate plasmin, although the parent compound, sodium flufenamate, above 5 mM totally inactivates plasmin (Fig. 6). In Table 3 it can also be seen that N-Flu-β-Ala was effective in the washed clot lysis assay in 36 hr at concentrations above 2 mM, while N-Flu-Glu was readily effective at 10 mM. It appeared that the ability of N-Flu-β-Ala and N-Flu-Glu to inactivate directly purified α2-antiplasmin corresponded to their activity in washed clot lysis assays.

Reversibility of Inactivation of α2-Antiplasmin by N-Flu-Glu

The reversibility of the direct inactivation of α2-antiplasmin by N-Flu-Glu was studied (Table 4). When α2-antiplasmin was preincubated with N-Flu-Glu, its ability to inhibit plasmin activity was lost. However, dialysis of the reaction mixture containing the fibrinolytic compound and α2-antiplasmin to remove the N-Flu-Glu restored the ability of α2-antiplasmin to inhibit plasmin. Thus, the inactivation of α2-antiplasmin by N-Flu-Glu is reversible.

Reversibility of Inactivation of Cl Inhibitor by N-Flu-Glu

The reversibility of the direct inactivation of Cl inhibitor by N-Flu-Glu was also studied (Table 5). When Cl inhibitor was preincubated with N-Flu-Glu, it lost its ability to inhibit kallikrein. However, dialysis of the reaction mixture containing N-Flu-Glu and Cl inhibitor restored most of the observed ability of Cl inhibitor to inhibit kallikrein. Thus, the inactivation of Cl inhibitor by N-Flu-Glu is reversible.

Table 4. Reversibility of Inactivation of α2-Antiplasmin by N-Flu-Glu

<table>
<thead>
<tr>
<th>α2-Antiplasmin + N-Flu-Glu</th>
<th>α2-Antiplasmin + buffer</th>
<th>Control without α2-antiplasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not Dialyzed</td>
<td>Dialyzed</td>
<td></td>
</tr>
<tr>
<td>0.045</td>
<td>0.003</td>
<td>0.050</td>
</tr>
</tbody>
</table>

*600 μl of α2-antiplasmin at 140 nM and 14 μl of N-Flu-Glu at 450 mM in H2O or H2O alone were added to a 10 x 75 mm glass tube and incubated at 37°C for 30 min. Four aliquots of 100 μl were removed from each tube and dialyzed against 1 liter of 40 mM Na phosphate, pH 7.0, 0.02% Na azide, 1 mM β-mercaptoethanol, at 4°C. 200 μl of each sample were also kept at 4°C without dialysis. At 19, 43, and 68 hr, the buffer was changed and aliquots were assayed for α2-antiplasmin activity by mixing 15 μl of each sample with 3 μl 0.5 M Tris-CI, pH 8.1, 0.15 M NaCl, 0.02% Na azide, adding 15 μl of plasmin at 27 μg/ml and incubating the mixture at room temperature for 30 sec. 70 μl of S-2251 at 0.48 mg/ml were then added. The mixture was placed in a narrow black-masked cuvette with a 1-cm path length and the change in absorbance at 405 nm/min was recorded. The values in the table were obtained after 43 hr.

Table 5. Reversibility of Inactivation of Cl Inhibitor by N-Flu-Glu

<table>
<thead>
<tr>
<th>Cl Inhibitor Activity (ΔA₄₀₅/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not Dialyzed</td>
</tr>
<tr>
<td>Cl inhibitor + N-Flu-Glu</td>
</tr>
<tr>
<td>Cl inhibitor</td>
</tr>
<tr>
<td>Control without Cl inhibitor</td>
</tr>
</tbody>
</table>

*200 μl of Cl inhibitor at 131 nM and 14 μl of N-Flu-Glu at 450 mM in H2O or H2O alone were incubated for 30 min at 37°C. Two aliquots of 80 μl of each reaction mixture were removed and dialyzed at 4°C in separate flasks containing 50 mM Na acetate-acetic acid, 70 mM NaCl, pH 5.3, 1 mM Na₄EDTA, 1 mM benzamidine HCl, 0.02% Na azide, and 50 μg/ml polybrene. 40 μl of each reaction mixture were also kept at 4°C without dialysis. After 24 hr of dialysis, Cl inhibitor activity was assayed as described in Materials and Methods.

DISCUSSION

Studies of possible mechanisms of action of Flu-HPA, a derivative of flufenamic acid, as a fibrinolytic agent were undertaken. The profibrinolytic activity of Flu-HPA in washed clot lysis assays was found to be dependent on plasminogen. Thus, Flu-HPA did not enhance clot lysis by direct disruption of fibrin or through a plasminogen-independent pathway. The hypothesis that Flu-HPA directly inactivates α2-antiplasmin, the major inhibitor of plasmin in plasma was tested using purified proteins. Plasmin activity was determined using 125I-fibrin plates or the spectrophotometric tripeptide, D-Val-Leu-Lys para-nitroanilide. The results showed that Flu-HPA directly inactivates purified α2-antiplasmin.

The ability of Flu-HPA to inactivate directly Cl inhibitor, a protease inhibitor of several plasminogen activators, was also studied. Cl inhibitor was isolated using a new procedure that employs DEAE-Sephadex and SP-Sephadex chromatography. Using purified Cl inhibitor, it was demonstrated that Flu-HPA directly inactivates the inhibitory activity of Cl inhibitor against plasma kallikrein and purified β-Factor XIa.

Flu-HPA by itself does not inhibit plasmin, kallikrein, or β-Factor XIa as assayed with spectrophotometric tripeptide substrates. This finding differs from the results obtained for orthothymotic acid and S-1623, α(isobutyl-4-cyclohexene-1-yl) propionic acid, which inhibit both plasmin and α2-antiplasmin, and also for other fibrinolytic compounds studied in which fibrinolysis is inhibited at high concentrations of the synthetic derivatives. Thus, Flu-HPA may be more specific in its mechanism of action than these other compounds. It was found that Flu-HPA does not liberate enzyme activity from preformed complexes of either α2-antiplasmin plus plasmin or Cl inhibitor plus kallikrein. These results do not support a previously proposed mechanism for the enhancement of fibrinolysis by synthetic compounds that involved either disso-
ciation of a plasmin–antiplasmin complex or liberation of plasminogen activator activity from an activator–inhibitor complex.

It has been reported that flufenamic acid binds to albumin. In our experiments, no effect of albumin up to a concentration of 4.3 mg/ml was observed. However, the effect of Flu-HPA on α2-antiplasmin was completely abolished at albumin concentrations of 21.5 mg/ml. These results are consistent with von Kaula’s observation that higher concentrations of synthetic compounds were required to lyse plasma clots into which synthetic compounds were incorporated rather than the lyse preformed hanging clots. He suggested that this was due to the binding of the fibrinolytic compounds to inert plasma proteins such as albumin during clot formation.

Two water-soluble derivatives of flufenamic acid, N-Flu-Glu and N-Flu-β-Ala, directly inactivate purified α2-antiplasmin. They do not inactivate plasmin up to the highest concentrations tested, 40 mM and 18 mM, respectively, as assayed with the spectrophotometric tripeptide substrate. It was also found that the fibrinolytic activities of these compounds in washed clot lysis assays correspond to their ability to inactivate α2-antiplasmin.

α2-Antiplasmin activity that has been abolished by treatment with N-Flu-Glu is restored after dialysis to remove N-Flu-Glu. Thus, the effect of this compound on α2-antiplasmin appears to be reversible. These results differ from those for the fibrinolytic compound, S-1623, which directly inactivates α2-antiplasmin but is not dissociated from α2-antiplasmin after extensive dialysis. Thus, N-Flu-Glu and S-1623 presumably do not interact with α2-antiplasmin through exactly the same mechanism.

The interaction of N-Flu-Glu with CI inhibitor appears to be reversible, since dialysis of a mixture of N-Flu-Glu and CI inhibitor to remove the compound restored the inhibitory activity of CI inhibitor.

The ability of flufenamic acid derivatives to inactivate directly both α2-antiplasmin and CI inhibitor demonstrates that these fibrinolytic agents can inactivate both the major inhibitor of plasmin in plasma and an inhibitor of plasminogen activators, since CI inhibitor inhibits activated Factor XII, activated Factor XI, and plasma kallikrein, all of which have been reported to activate plasminogen. The relative importance of these different activities of flufenamic acid derivatives in different fibrinolytic assays will depend on the design of the particular assay itself. Derivatives of flufenamic acid could be utilized in vitro assays or other procedures in which removal of the plasma protease inhibitors is desirable. Moreover, Flu-HPA is the first synthetic fibrinolytic agent tested that does not appear to inactivate plasmin. Hence, this property of the compound and related derivatives may make them especially useful for in vitro fibrinolytic or plasminogen activator assays.

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