Demonstration of a Fast-Acting Inhibitor of Plasminogen Activators in Human Plasma

By Egbert K.O. Kruithof, Chiên Tran-Thang, Adriana Ransijn, and Fedor Bachmann

This report describes a plasmatr, fast-acting, specific inhibitor (antiactivator) of tissue-type plasminogen activator (t-PA) and urokinase (UK). After addition of t-PA to human plasma, biexponential decay of activity occurred. The initial rapid inhibition of t-PA activity (half-life of approximately one minute) was correlated with the formation of a complex of a molecular weight of 110,000, suggesting a molecular weight in the order of 40,000 for the antiactivator. Diisopropylfluorophosphate (DFP)-inactivated t-PA did not form complexes with antiactivator. The

PROTEASE INHIBITORS are of vital importance in controlling the activity of proteases involved in processes of limited proteolysis, such as coagulation, fibrinolysis, complement activation, and inflammation.1,2 All known protease inhibitors present in human plasma interact with several proteases, but, in general, there is a primary inhibitor for each enzyme.2 It has been known for many years that plasmin could be inhibited by α2-macroglobulin, α1-protease inhibitor, antithrombin III, and C1'-inhibitor, but the observed inhibition rates were too slow to account for the rapid disappearance of plasmin activity in human plasma. The systematic search for a specific inhibitor of plasmin led to the discovery of α2-antiplasmin.3,4 In the concentrations present in human plasma, the latter inhibits plasmin with a half-life of less than one tenth of a second.6 Despite numerous reports dealing with the inhibition of plasminogen activators (PA) in human plasma and serum,7-10 specific, fast-acting inhibitors of tissue-type plasminogen activator (t-PA) or urokinase (UK) have not yet been convincingly demonstrated in human plasma.

Korninger and Collen reported that the activity of human t-PA, incubated in human plasma, decreased with a half-life of about 90 minutes.11 The slow inactivation of t-PA was associated with the formation of complexes of t-PA with α2-antiplasmin, α1-protease inhibitor, and α2-macroglobulin.11,12 In human plasma, Murano et al reported that high molecular weight UK (HMW-UK) at concentrations of 100 U/mL, decayed with a half-life of one hour and formed complexes with α2-macroglobulin and antithrombin III.13 Holmberg et al observed that HMW-UK at “low” concentrations complexed mainly with α2-antiplasmin and α2-macroglobulin.14 In view of the slow rate of inhibition of t-PA and UK, it appears unlikely that these protease inhibitors contribute to the physiologic regulation of PA activity in plasma.

In a preliminary report, we recently provided some evidence for the existence of a fast-acting inhibitor of t-PA and demonstrated that it forms complexes with t-PA and UK.15 In this article, we provide further data on its plasma concentrations, its molecular weight, the kinetics and mechanism of inhibition of t-PA, and on the formation of complexes of t-PA and UK with the fast-acting inhibitor and with other plasma protease inhibitors.

MATERIALS AND METHODS

Materials
Sodium citrate-citric acid anticoagulant, pH 4.5, was obtained from Behring-Hoechst, Zürich, Switzerland; heparin (Liquemen) and thrombin (Topostasin) were from Hoffmann-La Roche, Basel, Switzerland; fibrinogen (bovine, plasminogen-rich) from Poviet, Oss, The Netherlands; fibrinogen (Fibi-125 C, 75% clottable) from Sorin Biomedica, Saluggia, Italy; albumin (bovine, fraction V) from Miles, Elkart, IN; aprotinin (Trasylol) from Bayer, Leverkusen, Germany; agarose (low melting point) from BRL, Gaithersburg, MD; DFP from Fluka, Buchs, Switzerland; IODOGEN from Pierce, Oud Beijerland, The Netherlands; 1151 from New England Nuclear, Dreieich, West Germany; DEAE Affi-Gel Blue and all reagents for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad, Richmond, CA; dextran (Macrodex 6%), CNBr-Sepharose 4B and the molecular weight standard proteins for SDS-PAGE: phosphorylase b (94 kD), bovine serum albumin (67 kD), and ovalbumin (43 kD), from Pharmacia, Uppsala, Sweden; and myoglobin from Serva, Heidelberg, West Germany. Rabbit antiserum against human α2-antiplasmin and α2-macroglobulin were from Nordic Immunology, Tijburg, The Netherlands, and against human C1'-inhibitor, α1-protease inhibitor (antitrypsin), and antithrombin III from Behringwerke AG, Marburg, Germany.

From the Laboratoire Central d’Hématologie, CHUV, Lausanne, Switzerland.

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Address reprint requests to Dr Egbert K. O. Kruithof, Laboratoire Central d’Hématologie, CH-1011 Lausanne, Switzerland 021 41 52 77.

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Pure high molecular weight urokinase (UK) was a gift from Hypolob, Coinsins, Switzerland.

Blood was obtained from apparently healthy blood donors, anticoagulated by mixing nine parts with one part of citrate anticoagulant, placed in an ice bath, and centrifuged for 15 minutes at 3,600 g and 4 °C. The plasmas were stored at -70 °C.

**Purification of Porcine t-PA**

Pig heart tissue was delipidated and dried with acetone and extracted with potassium acetate as described before. After ammonium sulfate fractionation (0.51 to 2.54 mol/L), fibrin adsorption, affinity chromatography on zinc-chelate-Sepharose and on benzamidine-Sepharose, and ion-exchange chromatography on CM-Sepharose, a t-PA preparation was obtained that moved as a single band on reduced and nonreduced SDS-PAGE (approximately mol wt 68,000). By definition, this apparently pure t-PA was assigned a specific activity of 100,000 U/mg, in analogy to HMW-UK, which also has 100,000 U/mg.

**Radiolabeling of t-PA**

T-PA was radioiodinated using the technique of Fraker and Speck. Twenty micrograms of t-PA in 10 μL of 50 mmol/L sodium borate, pH 7.4, 500 mmol/L NaCl, 0.01% Tween 80, and 0.05% sodium azide was placed in a polypropylene tube coated with 50 μg lodogen. Ten microliters of low salt borate buffer (as above, but having 100 mmol/L NaCl), containing 1 mM of Na2131, was added and the tube incubated for ten minutes at 0 °C. The sample was diluted with 100 μL of the low salt borate buffer and passed over a 0.5-mL column of anti-t-PA-Sepharose (made by coupling 1 mg of rabbit anti-t-PA antibodies to CNBr-activated Sepharose 4B). The column was washed with 20 mL of low salt borate buffer, and t-PA was eluted with 0.1 mol/L glycine-HCl, pH 2.2, containing 0.01% Tween 80 and 0.5 mg/mL myoglobin. The eluate fractions were immediately neutralized by addition of 0.1 vol of 1 mol/L Tris. The radioiodinated t-PA had retained about 40% of its activity and had about 120 nCi 131I per unit of t-PA. DFP-inactivated 125I-t-PA (DIP-t-PA) was prepared by incubation for two hours at 25 °C with 5 mmol/L DFP.

**Recovery of PA From Noncrosslinked Plasma Clots**

Ten microliters of a solution of t-PA was added to 0.2 mL of human plasma and incubated at 37 °C for various time periods. Thereafter, 0.1 mL of a solution of thrombin (30 U/mL) in 50 mmol/L imidazole-140 mmol/L NaCl, pH 7.35, containing 300 U/mL aprotinin and 30 mmol/L EDTA was added. The clots were incubated at 37 °C for a further ten minutes and transferred to an ice bath. The fibrin was collected using a plastic spatula, dried on tissue paper, and washed for ten minutes in ice-cold water. The fibrin was then dissolved in 17 mmol/L acetic acid, and PA activity was measured on fibrin plates.

**Assay of PA Activity**

Thirty-microliter droplets of a suitably diluted sample was applied on the surface of a fibrin plate (1.2 g/L fibrin, 40 mg/L plasminogen, 12 g/L dextran, 10 mmol/L Ca2+ in 0.12 mol/L Tris-HCl, pH 7.8). After 15 hours of incubation at 37 °C, lysis zones were compared with those of a standard curve of a laboratory t-PA standard. An activity of 1 U/mL corresponds to an activator concentration of 10 ng/mL (0.15 nmol/L).

**t-PA Induced Lysis of 125I-Fibrin-Labeled Plasma Clots**

125I-fibrinogen was dissolved in 50 mmol/L imidazole-140 mmol/L NaCl, pH 7.35, containing 1% albumin. An aliquot of 10 μL of 125I-fibrinogen (0.4 × 106 cpm) and 10 μL of t-PA (200 U/mL) was added to 0.2 mL of plasma. After a preincubation time of 0.2, 5, or 10 minutes at 37 °C, 0.1 mL of a solution of thrombin (30 U/mL) and 30 mmol/L EDTA was added. The clots were further incubated for 40 minutes at 37 °C. The tubes were transferred to an ice bath and centrifuged for 15 minutes at 4,000 g and 4 °C. The radioactivity of the serum was determined and percent clot lysis calculated using the formula:

\[
\text{Percent clot lysis} = \frac{1 - (t - p)}{(t - a)} \times 100
\]

where \(t\) is total cpm of 125I-fibrinogen added, and \(p\) and \(a\) are cpm of 0.1-mL aliquots of serum prepared in the presence or absence, respectively, of t-PA, multiplied by 3.2 to correct for volume.

**Study of t-PA–Antiacтивator Complexes in Plasma**

SDS-PAGE was performed according to Laemmli, using a 7.5% polyacrylamide separating gel of 12.7 × 14 × 0.15 cm and a 4% stacking gel. The samples were diluted in SDS-PAGE sample buffer containing 0.125 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.02% bromophenol blue, but no reducing agent. Fifty microliters of diluted sample was applied on the stacking gel. After five hours of electrophoresis at 25 mA per gel, the gels were washed for one hour in 2.5% Triton X-100 and for ten minutes in deionized water. Fibrinolytic activity waszymographically revealed by placing the gels on a plasminogen-rich fibrin-agarose underlay. The observation that activator-inhibitor complexes show activity on fibrin-agarose underlays was exploited to study complex formation of t-PA or UK with antiaactivator and with other protease inhibitors. Ten microliters of t-PA or UK (200 U/mL) was added to 0.2 mL of plasma, incubated for various periods at 37 °C, and diluted tenfold in SDS-PAGE sample buffer prior to electrophoresis. To determine whether the active site of t-PA interacts with antiaactivator, we added 10 μL of radioiodabeled t-PA (20 U/mL, 2 μCi/mL) or an equivalent amount of DIP-t-PA to 50 μL of a plasma containing 8 U/mL of antiaactivator. The mixtures were incubated for ten minutes at 37 °C and diluted tenfold with SDS-PAGE sample buffer. After SDS-PAGE of 20-μL samples, the gels were stained in 2.2 g/L of Coomassie Brilliant Blue R 250 in acetic acid/methanol/H2O (1:4:4), destained in acetic acid/methanol/H2O (1:4:4), and dried on a Hoyer slab gel drying apparatus. Autoradiography was done for 40 hours on Kodak XAR-5 film, using a Kodak X-Omat intensifying screen (Kodak, Rochester, NY). The mobilities of t-PA or their complexes were compared with those of molecular weight marker proteins.

**Complex Formation of t-PA With Known Plasma Protease Inhibitors**

Immunoglobulin fractions were prepared from rabbit antiserum against α2-antiplasmin, Cl′-inhibitor, α2-macroglobulin, α2-protease inhibitor, and antithrombin III. One-half milliliter of antiserum was dialyzed against 20 mmol/L K2HPO4, pH 8.0, and passed over a 5-ml DEAE-Affigel Blue column. The immunoglobulins in the run-through peak (about 5 mg) were dialyzed against 100 mmol/L sodium bicarbonate-500 mmol/L NaCl, pH 8.1, and coupled to 0.3 mL CNBr-activated Sepharose. Over 90% of the protein bound to the Sepharose. t-PA (10 U/mL final) was added to antiaactivator-rich or -poor plasma and incubated for six hours at 37 °C. Aliquots were further incubated for 40 minutes at 37 °C. The tubes were transferred to an ice bath and centrifuged for 15 minutes at 4,000 g and 4 °C. The radioactivity of the serum was determined and percent clot lysis calculated using the formula:

\[
\text{Percent clot lysis} = \frac{1 - (t - p)}{(t - a)} \times 100
\]
(0.2 mL) of either of these plasmas were passed over each immunosorbent column. After washing, bound material was eluted in 0.1 mol/L glycine-HCl, pH 2.2, and immediately neutralized by the addition of one-tenth volume of 1 mol/L Tris. Run-through fractions and the eluates were diluted tenfold with SDS-PAGE sample buffer and, after electrophoresis, zymographically analyzed as described above.

RESULTS

Rate of Inhibition of t-PA in Human Plasma

Figure 1 shows that t-PA activity was quantitatively recovered from plasma clots when t-PA and thrombin were added simultaneously to the plasma [zero minute incubation time; recoveries were 98% ± 8% (mean ± SD, N = 4) and 94% ± 4% when fibrin-adsorbed t-PA was left in the clot environment for 10 and 30 minutes, respectively], demonstrating that clot-bound t-PA activity was relatively stable.

When t-PA was incubated in plasma for various periods prior to clotting, a biexponential decay of activity was observed, indicating that the inactivation of activity did not follow pseudo-first-order kinetics. In an antiactivator-rich plasma, the half-life of t-PA activity in the initial decay phase was about one minute, and after ten minutes, about 85% of the added t-PA activity had been inhibited. In another plasma, decay of t-PA activity was approximately two to three times slower, and activity losses after ten minutes were 40% to 45%. The initial rapid decay phase was followed by a much slower secondary decay phase, which was similar for both plasmas, and in which the half-life of t-PA activity was about 90 minutes. The rapid inhibition of t-PA activity in the primary inhibition phase was accompanied by the formation of a t-PA–inhibitor complex with an apparent mol wt of 110,000 (Fig 2B). The formation of this complex was hardly recognizable in plasmas that did not exhibit the rapid initial decay phase (Fig 2A).

The concentration of the fast-acting inhibitor of t-PA (antiactivator) was determined from the difference of total initial t-PA activity and that represented by the intercept of the secondary decay curve with the ordinate (Fig. 1). It was estimated to be 1.3 nmol/L in one plasma and 0.6 nmol/L in the other plasma. The two- to threefold difference in initial rate of inhibition, resulting from a twofold difference in antiactivator concentration, is consistent with second-order rate kinetics for the interaction of t-PA with antiactivator. Using the observation that half of 1.5 nmol/L t-PA was inhibited by 1.3 nmol/L antiactivator in about one minute (Fig 1), the second-order rate constant for the interaction was calculated to be in the order of 107 mol/L · sec−1.

Level of Antiactivator in Plasma of Normal Individuals

In a preliminary study, we added varying concentrations of t-PA to an antiactivator-rich plasma pool. The recovery of activity after a ten-minute incubation at
37 °C was 8% ± 1% (N = 4), 61% ± 12%, and 83% ± 3% at t-PA concentrations of 10, 20, and 40 U/mL, respectively, indicating that at higher concentrations of t-PA the antiactivator had become saturated.

Because the percentage decrease in activity was greatest, at 10 U/mL, we used this concentration to determine antiactivator levels in the plasma obtained from 20 resting, apparently healthy volunteers (all hospital personnel, eight females, 12 males, aged 19 to 46 years). In these plasmas, the inhibition of t-PA activity was very variable, ranging from 6% to 86% (mean 30%, SD 18%), suggesting antiactivator levels varying from 0.6 to 8.6 U/mL (0.1 to 1.3 nmol/L or about 5 to 60 ng/mL). Figure 3 shows that for ten plasmas selected at random, the degree of inhibition closely matched the extent of complex formation of t-PA with antiactivator.

**t-PA-Induced Lysis of 125I-Fibrin-Labeled Plasma Clots**

Because decreased recovery of t-PA could also occur if interaction of t-PA with a plasma factor decreased its binding to plasma clots, we developed an assay that allowed the direct measurement of the remaining fibrinolytic activity. t-PA was added to antiactivator-rich plasma containing 125I-fibrinogen. After preincubation periods of zero to ten minutes at 37 °C, thrombin was added. After a further 40-minute incubation of the clots, the percentage of released radiolabel was determined. Figure 4 demonstrates that the time-dependent decrease of activity recovered from the depolymerized clot at the end of the preincubation period correlated well with the decrease of the lysis of the radiolabeled plasma clot.

**Interaction of Radioiodinated t-PA and DIP-t-PA With Antiactivator**

In plasma, DIP-t-PA, in contrast to t-PA, failed to form complexes with antiactivator (Fig 5). This indicates that the active site of t-PA is essential for the complex formation. The smear of radioactivity observed below the 110,000 band suggests that, during electrophoresis, the complex of t-PA and antiactivator partly dissociated.

Fig 3. Correlation between inhibition of t-PA in plasma and the formation of a 110,000 mol wt complex of t-PA with antiactivator. t-PA (10 U/mL) was added to ten different plasmas and incubated for ten minutes at 37 °C. At the end of the incubation period, a small aliquot was diluted tenfold with SDS-PAGE sample buffer, electrophoresed, and t-PA activity zymographically revealed on fibrin-agarose underlays (top). The remainder of the plasma was clotted by the addition of thrombin-EDTA-trasylol and t-PA activity, recovered from the washed and acid-dissolved clots, was measured on fibrin plates (bottom).

Fig 4. Inhibition of the fibrinolytic activity of t-PA. t-PA (10 U/mL) was preincubated for zero to ten minutes in 0.2 ml of antiactivator-rich plasma to which 0.4 μCi of 125I-fibrinogen had been added. To determine t-PA-induced clot lysis (●●●●), the plasma was clotted by the addition of thrombin. After a further incubation of 40 minutes at 37 °C, clots were centrifuged and radioactivity measured in the serum. To determine the remaining t-PA activity at the end of the preincubation period (□□□□), the plasma was clotted with thrombin-EDTA-trasylol and t-PA recovered from the washed and acid-dissolved clots. (Results are mean ± SD, N = 4.)

Fig 5. Complex formation of t-PA and DIP-t-PA with antiactivator. The figure shows an autoradiographic analysis after SDS-PAGE. (A) 125I-t-PA; (B) 125I-t-PA added to antiactivator-rich plasma and immediately diluted with SDS-PAGE sample buffer; (C) as B but after ten-minute incubation at 37 °C; (D) incubation of DFP-inactivated 125I-t-PA for ten minutes at 37 °C in the same plasma.
Complex Formation of t-PA With Protease Inhibitors in Plasma

Figure 6 shows that after prolonged incubation (six hours) of t-PA in plasma, the 110-kD complex persisted in antiactivator-rich and antiactivator-poor plasma. In the former, this was the only complex detectable; in the latter, two further complexes had formed. One had a mol wt corresponding to the complex of t-PA and α2-antiplasmin (140,000) and bound to immobilized antibodies against α2-antiplasmin. A second had a mol wt compatible with a complex of t-PA and C1′-inhibitor (170,000) and bound to immobilized antibodies against C1′-inhibitor. The 110-kD complex was not retained by any of the immunosorbent columns to which antibodies against the other known major plasma protease inhibitors (α2-macroglobulin, α1-protease inhibitor, and antithrombin III) had been coupled.

Interaction of HMW-UK With Antiactivator

In addition to t-PA, plasma also contains UK. Figure 7 shows complex formation after the addition of HMW-UK to human plasma at a concentration of 10 U/mL. In antiactivator-poor plasma, three different complexes were clearly recognizable after a 20-minute incubation. The complex with the lowest mobility (approximately 155,000) bound to immobilized anti-C1′-inhibitor. A second complex, with a mobility of approximately 125,000, could be adsorbed to an anti-α1-antiplasmin column. A third complex (95,000) formed a faintly expressed lysis band on the zymogram. In antiactivator-rich plasma, rapid formation of a 95,000 complex of UK with antiactivator occurred. This complex was not adsorbed by immobilized antibodies to α1-protease inhibitor, α2-macroglobulin, antithrombin III, α2-antiplasmin, or C1′-inhibitor. Preincubation of antiactivator-rich plasma with an excess of t-PA prevented the formation of UK-antiactivator complexes and vice versa. The fast-acting inhibitor of t-PA thus is identical to that of UK. Radiolabeled DFP-inactivated UK, in contrast to radiolabeled UK, did not form complexes with antiactivator (data not shown).

DISCUSSION

In this article, we demonstrate that human plasma contains a fast-acting inhibitor of both t-PA and UK (antiactivator). In human plasma, t-PA activity decays in a biexponential fashion. The rapid primary inhibition phase is correlated with the formation of a t-PA-containing complex of a mol wt of 110,000. Addition of HMW-UK to plasmas having a high concentration of antiactivator leads, within a few minutes, to the formation of a complex with a mol wt of 95,000. The 40,000 increase is identical to that observed for t-PA. Preincubation of the plasma with an
excess of t-PA prevented complex formation of UK, and vice versa. These results demonstrate that, in human plasma, t-PA and UK rapidly form complexes with the same inhibitor. DFP-inactivated t-PA or UK did not form complexes, illustrating that the active site of t-PA and UK is essential for complex formation. The mechanism of inhibition by antiactivator is thus similar to that observed with other serine protease inhibitors, but it remains to be determined if, during the linkage of t-PA or UK to antiactivator, a small fragment is cleaved from antiactivator, as is the case for some other protease inhibitors, such as α₁-protease inhibitor.

The rate constant of the interaction of t-PA with antiactivator (of the order of 10⁹ mol/L⁻¹ sec⁻¹) approaches the upper theoretical limit of the rate constant of protein–protein interactions. In the secondary inhibition phase, t-PA had a half-life of about 90 minutes and formed complexes with Cl⁻-inhibitor and α₁-antiplasmin. These results are in agreement with other reports. However, we were unable to demonstrate complexes of t-PA with α₁-protease inhibitor or α₂-macroglobulin. The described experiments show that, in plasma, t-PA at physiologic concentrations preferentially forms complexes with antiactivator. Complex formation with other plasma protease inhibitors only occurs after prolonged incubation and if t-PA is added at high concentration (> 10 U/mL). The SDS-PAGE fibrin-agarose underlay technique was well suited to illustrate complex formation of t-PA and UK with antiactivator or other plasma protease inhibitors. The chemical basis of this phenomenon, which had already been described by the developers of the method, is at present unknown. It appears likely that active enzyme is recovered by partial decylation of the protease–inhibitor complex after denaturation by SDS and renaturation during the Triton X-100 washing procedure.

The inhibition of t-PA by antiactivator was considerably slower in a plasma clot environment. Preservation of clot-bound t-PA activity is reminiscent of the protective effect of fibrin on the inhibition of plasmin by α₁-antiplasmin. This observation thus adds a new element to the regulation of t-PA activity in plasma and in a clot environment. For our determinations of the inhibition rate of t-PA and of the plasma level of antiactivator, we made use of the relative stability of fibrin-bound t-PA. The results given in Fig 3 demonstrate the validity of our method to measure antiactivator in plasma.

The level of antiactivator in the plasma of apparently healthy blood donors was quite variable (range 0.1 to 1.3 nmol/L). A similar great variability is found in the level of t-PA activity after physiologic stimuli. These two phenomena are probably related to each other. SDS-PAGE of the euglobulins of normal “resting” volunteers followed by zymographic analysis on fibrin-agarose underlays always revealed the t-PA–antiactivator complex and rarely free t-PA. The intensity of the 110,000 mol wt band varied greatly in resting individuals, suggesting that baseline secretion of t-PA is quite different among individuals. Incidental release, prior to blood collection, of larger amounts of t-PA following a common stimulus of daily life, such as physical exercise, anxiety, anger, or smoking, will further add to variability of free antiactivator concentrations in plasma.

The evidence for and against the existence of a specific inhibitor of t-PA and UK in plasma was debated for a long time; some authors, even recently, denied its existence. The difficulty in detecting antiactivator activity in human plasma stems from its low concentration and from the lack of assays that measure directly and specifically t-PA activity in plasma. Thus, more solid evidence for the existence of a rapid inhibitor of t-PA in human plasma has only begun to accumulate over the last 12 months.

A fast-acting inhibitor of PAs having a mol wt of 47,000 has been partially purified from human placenta. Human fibroblasts produce a fast-acting inhibitor of UK, designated protease-nexin, which has a mol wt of about 50,000. A similar inhibitor has been described in the culture supernatant of endothelial cells and hepatoma cells. At present, it is not yet established whether or not these inhibitors bear any relationship to each other or to the antiactivator in plasma.

The observation that the described antiactivator inhibits physiologic concentrations of t-PA and of double-chain UK at a rapid rate suggests that the antiactivator plays an important role in the regulation of physiologic fibrinolysis.

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INHIBITOR OF PLASMINOGEN ACTIVATORS

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EK Kruithof, C Tran-Thang, A Ransijn and F Bachmann