



"Plasminogen activators having a reduced capacity to
bind lysine"

5 The invention relates to an advantageous use of plasminogen activators and claims the priority of German patent application 103 42 518.7, the content of which is hereby incorporated by reference.

10 The establishment of thrombolysis as a therapy option in connection with various thromboembolic diseases is regarded as having been largely concluded. The main interest in thrombolysis research is therefore directed toward the further development and modification of known thrombolytics and/or the improvement of
15 concomitant therapies.

A thrombolytic which is important for taking as a starting point for developing new thrombolytics is tissue plasminogen activator (t-PA), which has a higher
20 fibrin selectivity and a superior activity in comparison with streptokinase or urokinase. The deletion mutants reteplase and lanoteplase, as well as tenecteplase, are further developments of this plasminogen activator.

25 Tissue plasminogen activator (rt-PA) is a single-chain glycoprotein composed of 527 amino acids. The molecule which is initially single-chain (sct-PA) is cleaved by proteolysis into a two-chain form (tct-PA). t-PA
30 possesses defined structural and functional domains. Thus, the N-terminal constituent chain comprises the finger domain (F, Ser1-Lys49), the epidermal growth factor domain (E, Ser50-Asp87) and two kringle domains (K1, Thr88-Gly176; and K2, Asn177-Cys261). The C-
35 terminal chain, which includes a serine proteinase domain (P), comprises amino acids Ser262 to Pro527. By means of the amino acids His322, Asp371 and Ser478, the C-terminal chain forms the active center.



t-PA and its recombinant variants, and their preparation, are the subject, for example, of US patent 4,766,075 and a large number of publications (e.g. Bode and Renatus: Tissue-type plasminogen activator: variants and crystal/solution structures demarcate structural determinants of function, Current Opinion in Structural Biology 1997, 7: 865-872). A diagram of the structure of t-PA is enclosed as Fig. 1.

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Like most other trypsin-like serine proteinases, sct-PA is converted into the tct-PA form by cleavage. The cleavage takes place at the bond between Arg275 and Ile276. After that, the two chains are then only held together by way of a single disulfide bridge between serine 264 and serine 395.

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This numbering of the t-PA cleavage site corresponds to that chosen by Bode and Renatus (loc. cit). However, other authors use a different numbering as a basis and define the cleavage or activation site as R15-I16 or R310-I311. However, the sites which are defined in this way do not differ functionally or structurally.

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Tissue plasminogen activator (t-PA; alteplase) is able to activate plasminogen, which is transformed into plasmin. However, it is evident from kinetic constants that t-PA is only able to activate circulating plasminogen weakly. The two t-PA forms, i.e. both the single-chain molecule and the two-chain molecule, exhibit what are in principle the same pharmacological properties. However, fibrin-bound plasminogen is activated with a catalytic potency which is three powers of ten higher than that with which free plasminogen is activated. Accordingly, the thrombolytic property of tissue plasminogen activator is very greatly augmented by the presence of fibrin.

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A factor of from 500 to 1000 is reported for the relative fibrin selectivity of t-PA. The catalytic effect can also be increased by interaction of plasminogen activator with beta-amyloid or fibrinogen.

5 The ability of t-PA to be activated by fibrinogen is comparable to its ability to be activated by beta-amyloid.

The effect of plasminogen activator is controlled
10 physiologically by inhibitors, with plasminogen activator inhibitor I (PAI-1) being the important antagonist. The binding of PAI-1 to the light constituent chain of the t-PA molecule alters the structure of the catalytic center such that an
15 activation reaction with plasminogen can no longer take place (Bennet WF, Paoni NF, Keyt BA, Botstein D, Jones AJS, Presta L, Wurm FM, Zoller M: High resolution analysis of functional determinants in human tissue-type plasminogen activator. Journal of Biological
20 Chemistry 1991; 266: 5191-5201).

Tissue plasminogen activator is metabolized rapidly via the liver. Hepatic insufficiency in a patient therefore prolongs the plasma half-life of the substance
25 (Emeis JJ, van den Hoogen CM, Jense D: Hepatic clearance of tissue-type plasminogen activator in rats, Thomb Haemostas 1985; 54: 661-664; Tiefenbrunn AJ, Robison AK, Kurnik PB, Ludbrook PA, Sobel BE. Clinical pharmacology in patients with evolving myocardial
30 infarction of tissue plasminogen activator produced by recombinant DNA technology Circulation 1985; 71: 110-116).

Plasminogen activators are developed for treating
35 thrombotic diseases such as cardiac infarction and stroke. t-PA is currently the only thrombolytic which is approved by the Food and Drug Administration (FDA) in the USA for treating stroke.

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Nevertheless, suspicions have increased in the past that, while t-PA on the one hand exhibits the expected positive thrombolytic effects in connection with treating the stroke, it is on the other hand also responsible for an undesirable destruction of tissue. Thus, infusing t-PA into t-PA-deficient mice gave rise to larger infarctions (Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA: Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. Nat Med 1998; 4(2): 228-231). It is suspected that these enlarged lesions are due to stimulation of the NMDA-dependent glutamate receptors (Liberatore GT, Samson A, Bladin C, Schleuning WD, Medcalf RL: Vampire bat salivary plasminogen activator (desmoteplase): a unique fibrinolytic enzyme that does not promote neurodegeneration. Stroke 2003; 34(2): 537-543). A proteolytic cleavage of the NMDA receptor by t-PA could be responsible for this effect (Nicole O, Docagne F, Ali C, Margaille I, Carmeliet P, MacKenzie ET, et al: The proteolytic activity of tissue plasminogen activator enhances NMDA receptor-mediated signaling. Nat Med 2001; 7(1): 59-64).

The object of the present invention is therefore to provide a novel therapeutic treatment of thrombotic diseases, in particular of stroke.

This object is achieved by using a plasminogen-activating factor which exhibits a capacity to bind lysine which is reduced as compared with that of the native plasminogen activator. In a particularly advantageous embodiment, the plasminogen activator exhibits a modified kringle domain, preferably a kringle 2 domain which is modified as compared with that of the native t-PA. This latter domain can be entirely or partially deleted such that the capacity to bind lysine is reduced.

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The K2 domain, or regions which are functionally or structurally essentially homologous to it, can advantageously be modified such that lysine residues no longer bind or only bind with low affinity.

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In a particularly advantageous embodiment, the plasminogen activator which can be employed in accordance with the invention is modified t-PA.

- 10 The significance of the K2 domain, including the preparation of t-PA K2 deletion mutants, is described in detail in Horrevoets AJ, Smilde A, de Vries C, Pannekoek H (The specific roles of finger and kringle 2 domains of tissue-type plasminogen activator during in
15 vitro fibrinolysis: Journal of Biological Chemistry, 269, 17, 12639-12644, 1994).

- Because their ability to bind lysine is lacking or reduced, the plasminogen-activating factors which can
20 be used in accordance with the invention exhibit an elevated fibrin selectivity and a reduced ability to be activated by fibrinogen or beta-amyloid. This reduced ability to be activated by fibrinogen is probably the cause of the fibrin selectivity. The possession by the
25 plasminogen activator of a high fibrin selectivity is of considerable importance in connection with treating stroke, in particular, since, for example, the native t-PA can be activated by the fibrinogen which overcomes the damaged blood-brain barrier and then stimulates
30 glutamate-mediated excitotoxicity by subsequently activating the NMDA receptors. Accordingly, the reduction, in accordance with the invention, of the ability of the plasminogen activator to be activated by fibrinogen also leads to a reduction in neurotoxicity.

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The importance of the lysine-binding sites on the t-PA kringle 2 became clear, in particular, as a result of

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comparative investigations of the structural and functional domains of t-PA as compared with DSPA. DSPA is a plasminogen activator which was originally isolated from vampire bat saliva (in this regard, see
5 US patent 6,008,019; EP 0 383 417). DSPA can be isolated in four isoforms, of which DSPA α 1 can be prepared recombinantly using CHO cells.

In contrast to t-PA, DSPA only has one kringle domain.
10 This domain corresponds functionally and structurally more closely to the t-PA K1 domain than to the K2 domain and does not possess any lysine-binding sites (Bringmann P, Gruber D, Liese A, Toschi L, Krätzschar J, Schleuning WD, Donner P: Structural
15 features mediating fibrin selectivity of vampire bat plasminogen activators; Journal of Biological Chemistry 1995; 270(43): 25596-25603). There are therefore some statements in the literature to the effect that DSPA does not possess any kringle 2 domain.

20 Furthermore, DSPA is always present as a single-chain molecule since a plasmin activation site as in t-PA is lacking. As compared with t-PA, the activity of DSPA is stimulated about 45 000 times in the presence of fibrin
25 whereas, according to Gardell SJ, Duong LT, Diehl, York JD, Hare TR, Register RB, Jacobs JW, Dixon RA, Friedman PA (Isolation, characterization and c-DNA cloning of a vampire bat salivary plasminogen activator: Journal of Biological Chemistry 1989;
30 264(30): 17947-17952) this value is 205.

A diagram of the structure of DSPA is enclosed as Fig. 2. Fig. 2b shows a comparison of the amino acid sequence of t-PA with that of DSPA (SEQ ID Nos. 1+2).
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In the past, the ability of t-PA to bind fibrin was attributed functionally to the finger domain and kringle 2 (van Zonnenfeld AJ, Veerman H, Pannekoek H:

(1986) Proc. Natl. Acad. Sci. USA., 83: 4670-4674). However, more recent publications also suggest that the proteinase domain P may have a certain relevance in this regard (Bennett loc. cit.). The functions of the individual domains have also been investigated by Bakker AHF, Jacoline E., Weening-Verhoeffet. D., Verheijen JH (The role of Lysyl-Binding site of tissue type plasminogen activator in the interaction with a forming fibrin clod: Journal of Biological Chemistry 270, 21, 12355-12360, 1995).

Bakker et al. prepared various modifications of t-PA, for example two deletion mutants which lack either the kringle 2, the finger domain or the epidermal growth factor domain. These mutations were also combined and some of them were additionally provided with a point mutation in the kringle 2, namely involving the substitution D236N. This selective amino acid substitution leads to the replacement of the Asp in position 236 with asparagine and thereby to the deletion of the lysine-binding site (LBS in the K2 domain). For the preparation of the mutation, the reader is expressly referred to the abovementioned publication by Bakker et al including the references which are cited therein.

In their investigations, Bakker et al. demonstrated that occupation of the lysine-binding site in the K2 domain by EACA (ϵ -aminocaproic acid) markedly attenuated the binding of the native t-PA to fibrin. The same applied to the modification of the LBS by the D236N substitution except that the attenuation was less. Even the deletion mutant, which consisted solely of the K2 domain and the proteolytic C terminus, still bound to fibrin if only to a slight degree. It was only the K2P mutation, in which the LBS was deleted, which no longer exhibited any fibrin binding.

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While these results demonstrate, on the one hand, the importance of the F and K2 domains as well as the LBS in K2, they also demonstrate that the interaction of t-PA of fibrin is not mediated exclusively by the F and K2 domains. Rather, there is also the function of the LBS in the K2 domain, with the LBS presumably being responsible for stabilizing a conformation of the t-PA which is favorable for binding fibrin.

- 10 The importance of the K2 domain including its lysine-binding site has also been made clear by investigations carried out by Stewart RJ, Fredenburgh JG and Witz JI (Characterization of the interactions of plasminogen and tissue and vampire bat plasminogen activators with
15 fibrinogen, fibrin, and the complex of D-dimer noncovalently linked to fragment E: Journal of Biological Chemistry 273, 29, 18292-18299, 1998).

- In binding studies, Stewart et al. investigated, inter
20 alia, the affinities of t-PA and DSPA for fibrin and fibrinogen. In the studies, the affinities were investigated in the presence or absence of the lysine analog EACA in order to analyze the importance of the kringle-dependent interactions. They concluded, from
25 their investigations, that DSPA is unable to bind to fibrinogen because of the lack of the lysine-binding sites in the kringle. They therefore attribute a function, which is essential for the ability of t-PA to bind fibrin, to LBS in the t-PA kringle 2 in
30 combination with the finger domain.

- The experimental results reported in Example 1 confirm the different affinities of DSPA α 1 and recombinant human t-PA for the cofactors beta-amyloid (1-42),
35 fibrinogen and fibrin. To obtain these data, the kinetic parameters k_{cat} and K_m , and the k_{cat}/K_m ratio, were determined for each plasminogen activator/cofactor

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combination. In the absence of a cofactor, the efficiency of DSPAalphal for plasminogen activation was about 100 times lower than that of t-PA whereas the two compounds were equally efficient in the presence of fibrin. DSPAalphal was about 30 times less effective than t-PA in the presence of the fibrinogen or beta-amyloid cofactor. At a physiological plasminogen concentration of 2 μ M, these data give a ratio for the efficiency of fibrin as cofactor to that of fibrinogen or beta-amyloid as cofactor to be 480 in the case of DSPAalphal and 16 in the case of human t-PA.

As has already been explained above, the plasminogen-activating factor which is employed in accordance with the invention is characterized by the fact that its kringle 2 lysine-binding site is either absent or modified. In one embodiment, the kringle 2 is deleted. However, it is also possible, in another embodiment, to retain the kringle 2 and to substitute the aspartic acid at position 236 with asparagine. Accordingly, it is not crucial for the invention, according to the invention, of the modified t-PA for the kringle structure to be lacking but only for the lysine-binding site to be modified such that an interaction, which increases the activity of the t-PA, with a cofactor is no longer possible.

In another advantageous embodiment, the plasminogen activator, in particular the modified t-PA, which is used can be modified by the deletion of the kringle 1 such that the t-PA is no longer able to bind receptor. As a result, the tissue plasminogen activator is no longer metabolized in the native manner via the liver, resulting in the in-vivo half-life being prolonged (Rijken DC, Otter M, Kuiper J, von Berkel TJC: Receptor-mediated endocytosis of tissue-type plasminogen activator (t-PA) by liver cells. Thromb. Res. 1990; Supel. X: 63-71). Deletion of the kringle 1

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- is known from reteplase and described, for example, by Martin U, Bader R, Böhm E, Kohnert U, von Möllendorf E, Fischer S, Sponder G (BM 06.022: A Novel recombinant plasminogen activator. Cardiovascular drug reviews 5 1993; 11: 299-311). For this, use is made of mutants in which amino acids 4-176 are deleted. Prolonging the half-life makes it possible to administer the plasminogen activator as a bolus.
- 10 In a preferred embodiment, the plasminogen activator which can be used in accordance with the invention is based on one of the amino acid sequences shown in Figures 3, 11 or 13. Each of these amino acid sequences constitutes a modified t-PA in which the kringle 2 is
15 deleted and a structural change has been made in the region of the sequence for the t-PA activation cleavage site. This change can either only consist in the replacement of amino acids R and I in the activation cleavage site (e.g. by HS; see sequence SEQ ID No. 3,
20 Fig. 3) or additionally affect adjacent amino acids (e.g. replacement of FRIK with LHST in the sequence SEQ ID No. 4, Fig. 11). The latter modification corresponds to the structure of native DSPA at this point.
- 25 According to the invention, preference is given to producing, and employing for stroke treatment, a plasminogen activator which, particularly suitably, combines the advantages of native t-PA, namely, in particular, low immunogenicity when used in human
30 patients, with the advantages of DSPA, namely the lack of neurotoxicity. In one embodiment of the invention, the deletion of the t-PA kringle 2 is accordingly selected such that a transition region to the downstream structure is created, which transition region corresponds to the
35 comparable structure in DSPA. The transition region between the remaining kringle 1 domain of the modified t-PA and the downstream cysteine bridge is accordingly advantageously formed by the sequence SKAT. In DSPA,

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the sequence SKAT is located between the kringle and the cysteine bridge. This advantageous structure is realized, for example, in the sequence SEQ ID No. 4 as shown in Fig. 11.

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Fig. 12 (Multiple Sequence Alignment) shows a comparison between t-PA and the two described embodiments in accordance with the invention.

10 In another embodiment according to the invention, use is made of a plasminogen activator as depicted in Figure 13 (SEQ ID No. 5).

It is naturally also possible, in accordance with the invention to use proteins which possess sequences which are homologous with, or partially identical to, the amino acid sequences depicted in Figures 3, 11 and 13. Preference is given to homologies or identities of at least 70, preferably between 80 and 95%. These homologous or identical proteins exhibit the activity of a plasminogen-activating factor (preferably displayed as the release of pNA) and cause a blood clot to the lyse in vitro (see Example 3).

25 The plasminogen activator which can be used in accordance with the invention can be characterized by the lack of the finger and epidermal growth factor domains. This deletion substantially reduces binding to the liver receptors and once again prolongs the half-life (Larson GR, Timony GA, Horgan PG, Barone KM, Henson KS, Angus LB, Stoudemire JB: Protein engineering of novel plasminogen activators with increased thrombolytic potency in rabbits relative to activase. Journal of biological Chemistry 1991; 266: 8156-8161; Smalling RW: Molecular biology of plasminogen activators: what are the clinical implications of drug design? Am J Cardiol. 1996; 78 (suppl. 12): 2-7).

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Aside from deletion mutants, the plasminogen activators which are employed in accordance with the invention can also be only punctately modified by means of site-directed mutagenesis. Thus, it is known, for example, from rt-PA-TNK (tenecteplase) that three mutations at the binding site for the plasminogen activator inhibitor, on the one hand, and at the binding site by which t-PA binds to liver cells, on the other hand, lead to the catalytic activity being increased while, at the same time, the ability to be inactivated by PAI is decreased. This thereby gives tenecteplase a catalytic conversion constant K_{cat}/K_m which is increased 100-fold (in this regard, see also Paoni NF, Keyt BA, Refino CJ, Chow AM, Nguyen HV, Berleau LT, Badillo J, Pena LC, Brady K, Wurm FM, Ogez J, Bennett WF: A slow clearing fibrin-specific, PAI resistant variant of t-PA (T103N, KHRR 296-299 AAAA). Thromb Haemostas 1993; 70: 307-312 and Cannon CP, Love TW, McCabe CH, Kirshenbaum JM, Henry T, Sequira R, Schweifer M, Breed J, Cutler D, Tracy R, for the TIMI investigators. TNK-tissue plasminogen activators in myocardial infarction (TIMI) 10: Results of the initial patients in the TIMI 10 pilot - a phase 1, pharmacokinetics trial. Circulation 1995; 92 (suppl.): 1-415).

EXAMPLE 1:

1. Comparative analysis of the efficiencies of beta-amyloid (1 to 42), fibrinogen and fibrin as cofactors for plasminogen activation by DSPAalpha1 and recombinant human t-PA.

Materials and methods

The following substances were used:

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- 5 - DSPAalpha1 (prepared by Paion, batch No. 2DSA01,
 12 December 2002, sample 10). 10 mg of the
 substance were dissolved in 10 ml of sterile water
 in order to obtain a final concentration of
 1 mg/ml.
- 10 - Recombinant t-PA (Actilyse®, prepared by Paion,
 batch No. 102572). 10 mg were dissolved in 10 ml
 of sterile water to give a final concentration of
 1 mg/ml.
- 15 - Human Glu-plasminogen, purified from human plasma
 (prepared by Paion) and dissolved in PCLA buffer
 at a concentration of 25 µM.
- 20 - Human fibrinogen, essentially plasminogen-free,
 was obtained from Sigma (catalog No. F4883, batch
 12K7620). 25 mg were dissolved in 25 ml of PCLA
 buffer to give a final concentration of 1 mg/ml.
- 25 - Human thrombin (Sigma; catalog No. T7009. Batch
 61K7603). 100 U were dissolved in 10 ml of PCLA
 buffer to give a concentration of 10 U/ml.
- 30 - Flavigen.pli color reagent (D-but-CHT-Lys-p-
 nitroaniline-DHCL) from Biopool (catalog
 No. 101353, batch 1512016). 100 µM were dissolved
 in 50 ml of PLCR buffer to give a final
 concentration of 2 millimolar.
- 35 - beta-Amyloid protein (1-42) from Bachem (catalog
 No. H-1368, batch 0535120). 4 mg were dissolved in
 4 ml of 0.1 percent ammonium hydroxide to give a
 final concentration of 1 mg/ml.

All reagents were divided into aliquots and stored at
-20°C for no more than two weeks.

PLC Buffer (Jones 1990): 0.1 M NaCl.2H₂O (Acros catalog No. 20779); 0.03 M NaHCO₃ (Acros catalog No. 21712); 4 mM KCl (Fluka, catalog No. 60130); 1 mM CaCl₂.2H₂O (Acros, catalog No. 207780); 1 mM Na₂HPO₄.2H₂O (Fluka, catalog No. 71638); 0.3 mM MgCl₂.6H₂O (Acros, catalog No. 197530); 0.4 mM MgSO₄.7H₂O (Fluka, catalog No. 63140); 20 mM HEPES (Applichem, catalog No. A1969); 0.01% Polysorbate 80 (Fluka, catalog No. 93781).

10 Plasminogen activation assay:

The assay for plasminogen activation was carried out on microtiter plates at a total volume of 0.15 ml, as described by Bringmann et al. (loc. cit.). The reagents were added as follows:

50 µl of plasminogen (0-24 µM); 15 µl of cofactor (1 mg/ml); 10 µl of plasminogen activator 7.5 nM and 75 µl of Flavigen (2 mM).

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The final concentrations were as follows:

plasminogen activator: (t-PA or DSPAα1): 0.5 nM; Glu-plasminogen; 0.0625, 0.125, 0.25, 0.5, 1, 2, 4 and 8 µM; FlavigenPli: 1 mM and cofactors: 100 µg/ml.

25

The cofactors beta-amyloid (1-42), fibrinogen and fibrin were compared with the control without cofactor. For the control, the reaction mixture contained a further 0.13 units of human thrombin/ml.

30

Directly after the addition, the microtiter plate was introduced, at 25°C, into a molecular device ThermoMax microplate reader.

35

Stirring was carried out from time $t = 0$.

The optical density at 405 nm and 490 nm was measured at regular intervals. The optical densities at 490 nm were subtracted from the optical densities at 405 nm in order to eliminate the differences due to the movement of the liquid. All the experiments were carried out in three repeats using each plasminogen concentration and plasminogen activator concentration.

The K_m and k_{cat} values were determined (Figs. 4-6).

10

Figures 4

- a) Determining the molar extinction of the pNA.
- 15 b) Conversion of the Flavigen.pli by 10 nM plasmin as a function of the Flavigen.pli concentration (in μM).
- c) Michaelis-Menten plot of the conversion of
20 Flavigen by 10 nM plasmin. The initial rates from Fig. 4b were converted into pNA/s.

Figures 5

- 25 Curves of the optical density against the time for four different combinations of plasminogen activators and cofactors. Plasminogen concentrations of 8; 4; 2; 1; 0.5; 0.25; 0.125 and 0.0625 μM were used for this purpose.
- 30 a) t-PA without cofactor
- b) t-PA with beta-amyloid (1-42) as cofactor
- 35 c) t-PA with fibrinogen as cofactor
- d) t-PA with fibrin as cofactor

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e) DSPAalphanal without cofactor

f) DSPAalphanal with beta-amyloid (1-42) as cofactor

5 g) DSPAalphanal with fibrinogen as cofactor

h) DSPAalphanal with fibrin as cofactor

Figures 6

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Plot of $a \{= k_s \cdot k_{cat} \cdot PA.E.P. / (K_m + P)\}$ against the plasminogen concentration

15 a) Plots for t-PA without cofactor or beta-amyloid, fibrinogen or fibrin as cofactor

b) Plots for DSPAalphanal 11.38 without cofactors

Results:

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Table 1

	$k_{cat} (s^{-1})$	$K_m (\mu M)$	k_{cat}/K_m
t-PA ctrl	n.d.	n.d.	1272 ± 224
t-PA amyl	n.d.	n.d.	9307 ± 579
t-PA fbg	n.d.	n.d.	10204 ± 1546
t-PA fibrin	0.351 ± 0.35	0.178 ± 0.017	$1\ 990\ 000 \pm 327\ 000$
DSPA ctrl	n.d.	n.d.	12.5 ± 0.9
DSPA fbg	$1.649 \pm 0.110e-3$	2.465 ± 0.426	690 ± 181
DSPA amyl	$0.957 \pm 0.199e-3$	0.853 ± 0.316	1304 ± 785
DSPA fibrin	0.532 ± 0.136	1.098 ± 0.382	$506\ 000 \pm 120\ 000$

25 Table 1: Values for k_{cat}/K_m for the activation of plasminogen by recombinant human T-PA or DSPAalphanal using beta-amyloid (1 to 42), fibrinogen or fibrin as cofactors.

The results are given as the mean $\pm$ standard deviation of three independent experiments.

It was not possible to determine the precise values for k_{cat} and K_m since the K_m values were higher than the maximum plasminogen concentration ($8 \mu M$) which it was possible to use.

The derivation of the kinetic constants is not on its own sufficient for comparing the efficiencies of the different cofactors since the reaction rates at plasminogen concentrations close to or below the K_m values are dependent on the plasminogen concentration. For this reason, the reaction rates are calculated, using Michaelis-Menten kinetics, at physiological plasma plasminogen concentrations at $2 \mu M$. The relative efficiencies of the different cofactors for plasminogen activation by t-PA and DSPA α 1 (Table 2) are determined on this basis.

20

Table 2

cofactor	DSPA α 1	t-PA
none	1	101.8 ± 17.9
DSPA amyloid	27.3 ± 7.7	744.6 ± 23.6
DSPA fbg	29.8 ± 5.0	816.3 ± 123.7
DSPA fibrin	$13\ 560 \pm 2360$	$12\ 880 \pm 1360$

Relative rates of plasminogen activation at the physiological plasminogen concentration of $2 \mu M$, as calculated using the kinetic parameters in Table 1. The combination of the DSPA control is 1.

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EXAMPLE 2:

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The prion diseases, i.e. the spongiform encephalopathies, are fatal neurodegenerative phenomena which are characterized by an accumulation of the

abnormal isoform of the prion protein (PrP^{Sc}) in amyloid deposits. The normal prion protein (PrP^C) is expressed in many tissues, in particular in the brain, in which it is concentrated in the synapses. The mechanism of the change in the conformation of PrP^C to give PrP^{Sc} is still not well understood and nor is the mechanism which leads to neurodegeneration by way of PrP^{Sc} formation. A possible mechanism could lie in an effect on PrP^{Sc} in connection with the activation of plasminogen by t-PA.

We have already shown, using a recombinant protein, that PrP can increase t-PA-catalyzed plasminogen activation 300-fold (Ellis V, Daniels M, Misra R, Brown DR (2000): Plasminogen activation is stimulated by prion protein and regulated in a copper-dependent manner, Biochemistry 41: 6891-6896).

The ability of PrP to stimulate plasminogen activation was dependent on the conformation of the PrP, with the conformation being influenced by the presence or absence of Cu²⁺. The latter form contains a large number of beta structures which correspond to the PrP^{Sc} isoform and stimulate plasminogen activation. These data agreed with earlier observations that, while plasminogen bound to PrP^{Sc} which was isolated from scrapie-infected mouse brains, it did not bind to PrP^C as normal brain cells (Fischer M, Roeckl C, Rarizek P, Schwarz HP, Aguzzi A (2000): Binding of disease-associated prion protein to plasminogen. Nature 408: 479-483).

Furthermore, our experiments have shown that the mechanism of PrP-stimulated plasminogen activation also encompasses the binding of t-PA to apoPrP (i.e. PrP without Cu²⁺). The structures which are required for this binding have not yet been evaluated in detail. However, the binding is specific since it was prevented by DFP-inactivated t-PA.

Other methods:

The PrP batches which were used in these investigations were in each case derived from recombinant murine PrP isolated from *E. coli*. Following the purification of the His-labeled PrP, the protein folds once again, in the absence or presence of copper chloride, in order to form either a holo-PrP or an apo-PrP. Aggregated forms of these proteins are in each case produced by rapid dissolution in water. The protein was collected by centrifugation. The material is resistant to proteinase K digestion. A mutant $\Delta 51-90$ form, which does not comprise the Cu^{2+} binding site, was prepared in the same way. The effect of these different forms of PrP on plasminogen activation by DSPA was investigated by incubating the PrP (0-100 $\mu\text{g}/\text{m}$) with Lys-plasminogen (25 nm) and DSPA (0.25 nm). Plasminogen activation is determined by hydrolysis of the plasmin-specific dye H-d-Val-Leu-Lys-7-amido-4-methylcoumarin at 37°C using the SPECTRAMax Gemini-lourescence microplate reader. The controls of these experiments contain sc-t-PA, as a positive control for the effect of the PrP on plasminogen activation, and fibrin fragments, as a positive control for stimulation of the t-PA and DSPA activities.

The specificity of the plasminogen binding was determined by competition with DFP-inactivated t-Pa. The lysine analog EACA was also used as a competitive inhibitor.

Results:

The results of the experiments are presented in Table 3 and Figs. 7 to 10.

- 20 -

Fig. 7, in particular, provides informative insight. This figure makes clear that PrP specifically only activates t-PA and not DSPA. It was furthermore made clear that the lysine-binding site of the kringle 2 must, as the essential difference between DSPA and t-PA, be involved. Table 3

Reaction rates, M-ls-1

		minus PrP	plus PrP	PrP + Hep	fibrin
PrP preparation #1					
	tPA	7200	1 800 000		2 000 000
	DSPA	15.3	9.77		2 000 000
PrP preparation #2					
	tPA	7020	624 000	2 912 000	
	DSPA	15.75	162.75	5 565	

10 Fold stimulation

		minus PrP	plus PrP	PrP + Hep	fibrin
PrP preparation #1					
	tPA	1	250		2 777 778
	DSPA	1	0.638562		130 719
PrP preparation #2					
	tPA	1	88.88889	414.8148	
	DSPA	1	10.33333	353.3333	

EXAMPLE 3:

- a. **Preparing and purifying humanized DSPA (humDSPA)**
 (amino acid sequence as depicted in Fig. 11)

Recombinant human DSPA baculovirus DNA was prepared using the Bac-to-Bac system (Invitrogen) and the purified virus DNA was transfected into Sf9 insect cells. The recombinant baculovirus which was produced was amplified using Sf9 cells. HumDSAP was expressed using High Five insect cells in serum-free medium in suspension culture. The cell culture supernatant

containing the recombinant protein was frozen at -20°C until it was subjected to further working up.

5 The cell culture supernatant was thawed slowly at RT on a shaker and then centrifuged at $4000 \times g$ for 1 hour. The protein solution (1.5 l) was equilibrated to pH 6.0 with 50 mM ammonium acetate, loaded onto an SP-sepharose XL (Amersham) ion exchange column (350 ml bed volume) and washed with 5 column volumes of 50 mM
10 ammonium acetate solution. The bound protein was eluted in a gradient of 0-100% 1M ammonium acetate, pH 6, over 10 column volumes.

HumDSPA-containing fractions were identified by Western
15 blotting; following protein determination, these fractions were subjected to an activity test, frozen at -80°C and subsequently lyophilized.

In contrast to the standard activity assay, 50 μl of
20 eluate (= humDSPA-containing fraction), 50 μl of 0.2 M Tris, pH 8.0, and 100 μl of 2 mM S-2288 in PBS were used in this experiment.

b. Assay of DSPA activity

25 The activity assay employed is the determination of the rate at which a plasminogen activator converts the colorless substrate S-2288 into a colored product. The assay is a standard method for determining the
30 proteolytic activity of plasminogen-activating factors. The activity is visualized by determining the release of a chromogen (p-nitroaniline, pNA) by the test substance. The Chromogenix company offers the chromogen S-2288 for this purpose.

35 In this assay, the rate of reaction is not determined directly (e.g. in catal units); instead, comparison is made with a standard which is defined as 100% activity.

The reaction rate was measured in a buffer having the following composition: 25 mM Tris-HCl, pH 8/0.1% albumin/100 mM NaCl/1.1 mM glycine/1.2 mM mannitol/2.5 mM S-2288.

The measurement was carried out in a 96-well plate using blank values, standards and samples containing different concentrations of protein.

10

The increase with time in the absorption at 405 nm was recorded using a photometer. The slope of the linear portion of the curve was determined using Excel. This gives the activity.

15

Fig. 14 shows the activities of the fractions (expressed in S-2288 activity). The silver-stained gels can be seen in Figs. 15a and 15b. Figs. 16a and 16b show the Western blots. The protein detected in the Western blot corresponds to the protein marked with the arrowhead in the silver-stained gel.

20

[L = load; T=flow-through; W = wash; 0-40% B, A2, A3 ... fractions]

25

EXAMPLE 4:

Clot lysis (thrombolytic activity)

The residue of the humDSPA-containing fraction B6 was dissolved in 15 ml of PBS and the solution was centrifuged at 4000 x g for 15 min. This fraction was selected since it exhibited the highest purity. An aliquot was removed and used for the activity determination. The catalytic activity of this sample was roughly comparable to the catalytic activity of 5 µg of activase/ml.

30

35

The blood clots which were used were derived from a normal blood sample taken 24 h earlier and were in each case formed from 2 ml of blood which was transferred into polypropylene tubes and coagulated under natural
5 conditions. The lysis was carried out in PBS.

The humDSPA-containing fraction B6 (from the SPXL-sepharose capture step) was first of all lyophilized. The residue was dissolved in 15 ml of PBS and
10 centrifuged at 4000 g for 15 min. A sample of this solution was removed for an S-2288 activity determination before the blood clot was added. The catalytic activity of this sample corresponded essentially to the catalytic activity of 5 µg of
15 activase/ml.

Figures 17a to 17d show the results of the chronological course of the clot lysis at 0 h, 3 h, 4 h and 24 h after adding the humDSPA. The left-hand
20 experimental assay in each case shows the control. PBS containing humDSPA in the above-described quantity is present in the right-hand assays.

The blood cells slowly sediment out of the
25 disintegrating clot. A type of mesh, which can already be discerned after 4 h, remains after 24 h. In the assay, detached blood cells were aspirated after 4 h using a syringe in order to view the structure of the remaining clot.

30 Consideration has to be given to the fact that the fibrinolysis slows down when the clot is no longer hanging in its own lysate. This is the reason why any clot at all is still present after 24 h.

35 It is not possible to quantify this reaction. However, the humDSPA exhibits a marked increase in activity as compared with native DSPA since at least 4 times the

quantity of DSPA has to be used in order to dissolve a
clot of the same size.

Patent claims

1. The use of a plasminogen activator which has a lysine-binding capacity which is reduced as compared with that of the native t-PA for treating thrombotic diseases, in particular stroke.
5
2. The use as claimed in claim 1, **characterized by a** modification of the plasminogen activator at kringle 2 or domains which are functionally or structurally homologous therewith.
10
3. The use as claimed in claim 2, **characterized by a** deletion of kringle 2 or domains which are structurally or functionally homologous therewith.
15
4. The use as claimed in claim 2, **characterized by a** modification of the kringle 2 lysine-binding sites or domains which are structurally or functionally homologous therewith.
20
5. The use as claimed in claim 4, **characterized by** the substitution D236N or of amino acids which are homologous therewith.
25
6. The use as claimed in one of the preceding claims, **characterized in that** the plasminogen activator exhibits an homology of at least 75% with t-PA.
- 30 7. The use as claimed in claim 6, **characterized in that** the t-PA activation site is modified by deletion or substitution such that no proteolytic cleavage takes place.
- 35 8. The use as claimed in claim 7, **characterized by a** modification of the amino acid position R15-I16.

- 26 -

9. The use as claimed in claim 8, **characterized in that** the activation site exhibits an LHST sequence.
- 5 10. The use as claimed in one of the preceding claims, **characterized in that**, in the transition region between the kringle and the cysteine bridge, the plasminogen activator exhibits an amino acid sequence segment containing SKAT.
- 10 11. A plasminogen activator comprising an amino acid sequence as depicted in one of the Figures 3, 11 and 13.
- 15 12. A plasminogen activator, **characterized by** an identity of at least 70%, preferably an identity of from 80 to 90%, particularly preferably an identity of 95%, with a plasminogen activator as claimed in claim 10.
- 20 13. A plasminogen activator as claimed in one of claims 10 to 12, **characterized by** a thrombolytic activity which is increased as compared with that of the native DSPA.