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"Non-neurotoxic plasminogen activating factors for treating of stroke"

The invention pertains to the therapeutic use of non-neurotoxic plasminogen activators especially from the saliva of *Desmodus rotundus* (DSPA) preferentially for the treatment of stroke.

Different clinical pictures are summarized under the term "stroke" which correlate in their clinical symptoms. According to the respective pathogenesis a first differentiation between these clinical pictures in so called ischaemic and haemorrhagic insults is possible.

Ischaemic insults (ischaemia) are characterized in a reduction or interruption of the blood circulation in the brain due to a lack of arterial blood supply. Often this is caused by thrombosis of an arteriosclerotic stenosed vessel or by arterio arterial, respectively, cardiac embolisms.

Haemorrhagic insults are based inter alia on the perforation of brain supplying arterias damaged by arterial hypertonia. However, only approximately 20 % of all cerebral insults are caused by haemorrhagic insults. Thus, stroke due to thrombosis is much more relevant.

In comparison to other tissue ischaemias the ischaemia of the neuronal tissue is widely accompanied by necrosis of the effected cells. The higher incidence of necrosis in neuronal tissue can be explained with the new understanding of the phenomenon "excitotoxicity" which is a complex cascade comprising a plurality of reaction steps. The cascade is initiated by ischaemic neurons affected by a lack of oxygen which then lose ATP instantaneously and depolarize. This results in an increased postsynaptic release of the neurotransmitter glutamate which activates membrane bound glutamate receptors regulating cation channels. However, due to the increased glutamate release glutamate receptors become over activated.

Glutamate receptors regulate voltage dependent cation channels which are opened by a binding of glutamate to the receptor. This results in a Na^+ and Ca^{2+} influx into the cell massively disturbing the Ca^{2+} dependent cellular metabolism. Especially the activation of the Ca^{2+} dependent catabolic enzymes could give reason to the subsequent cell death (Lee, Jin-Mo et al., "The changing landscape of ischaemic brain injury mechanisms"; Dennis W. Zhol "Glutamate neurotoxicity and diseases of the nervous system").

Although the mechanism of glutamate mediated neurotoxicity is not yet entirely understood it is agreed upon that it contributes in a large extent to the neuronal cell death following cerebral ischaemia (Jin-Mo Lee, et al.).

Besides safeguarding vital functions and stabilizing physiological parameter the re-opening of the closed vessel has priority in the therapy of acute cerebral ischaemia. The re-opening can be performed by different means. The mere mechanical re-opening, as e.g. the PTCA after heart attack, so far has not yet led to satisfying results. Only with a successful fibrinolysis an acceptable improvement of the physical condition of patients can be achieved. This can be accomplished by a local application using a catheter (PROCAT, a study with pro-urokinase). However, despite first positive results this method has not yet been officially approved as a pharmaceutical treatment.

The naturally occurring fibrinolysis is based on the proteolytic activity of the serine protease plasmin which originates from its inactive precursor by catalysis (activation). The natural activation of plasminogen is catalyzed by the plasminogen activators u-PA (urokinase type plasminogen activator) and t-PA (tissue plasminogen activator) occurring naturally in the body. In contrast to u-PA, t-PA forms a so called activator complex together with fibrin and plasminogen. Thus, the catalytic activity of t-PA is fibrin dependent and is enhanced in its presence approximately 550-fold. Besides fibrin also fibrinogen can stimulate t-PA mediated catalysis of plasminogen to plasmin - even though to a smaller extent. In the presence of fibrinogen the t-PA activity is only increases 25-fold. Also the

cleavage products of fibrin (fibrin degradation products (FDP)) are stimulating t-PA.

Early attempts of thrombolytic treatment of acute stroke go back to the 1950s. First extensive clinical trials with streptokinase, a fibrinolytic agent from beta-haemolysing streptococci, started only in 1995. Together with plasminogen streptokinase forms a complex which catalyzes other plasminogen molecules into plasmin.

The therapy with streptokinase has severe disadvantages since it is a bacterial protease and therefore can provoke allergic reactions in the body. Furthermore, due to a former streptococci infection including a production of antibodies the patient may exhibit a so called streptokinase resistance making the therapy more difficult. Besides this, clinical trials in Europe (*Multicenter Acute Stroke Trial of Europe (MAST-E)*, *Multicenter Acute Stroke Trial of Italy (MAST-I)*) and Australia (*Australian Streptokinase Trial (AST)*) indicated an increased mortality risk and a higher risk of intracerebral bleeding (intracerebral haemorrhage, ICH) after treating patients with streptokinase. These trials had to be terminated early.

Alternatively, urokinase - also a classical fibrinolytic agent - can be applied. In contrast to streptokinase it does not exhibit antigenic characteristics since it is an enzyme naturally occurring in various body tissues. It is an activator of plasminogen and independent of a co-factor. Urokinase is produced in kidney cell cultures.

Extensive experience on therapeutic thrombolysis is available for the tissue type plasminogen activator - the so called rt-PA - (see EP 0 093 619, US 4,766,075), which is produced in recombinant hamster cells. In the 90s several clinical trials were performed world-wide using t-PA - with acute myocardial infarction as the main indication - leading to partially non-understood and contradictory results. In the so called *European Acute Stroke Trial (ECASS)* patients were treated within a time frame of 6 hours after the onset of the symptoms of a stroke intravenously

with rt-PA. After 90 days the mortality rate as well as the Barthel-Index were examined as an Index for the disability or the independent viability of patients. No significant improvement of the viability was reported but an - even though not significant - increase of mortality. Thus, it could be concluded, a thrombolytic treatment with rt-PA of patients being individually selected according to their respective case history immediately after the beginning of the stroke could possibly be advantageous. However, a *general* use of rt-PA within the time frame of 6 hours after the onset of stroke was not recommended since an application during this time increases the risk of intracerebral haemorrhage (ICH) (C. Lewandowski C and William Barsan, 2001: Treatment of Acute Stroke; in: Annals of Emergency Medicine 37:2; S. 202 ff.).

The thrombolytic treatment of stroke was also subject of a clinical trial conducted by the *National Institute of Neurologic Disorder and Stroke* (so called NINDS rtPA Stroke Trial) in the USA. This trial concentrated on the effect of intravenous rt-PA treatment within only three hours after the onset of the symptoms. Patients were examined three months after the treatment. Due to the observed positive effects of this treatment on the viability of patients, rt-PA treatment within these limited time frame of three hours was recommended although the authors found a higher risk for ICH.

Two further studies (ECASS II Trial: *Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischaemic Stroke* (ATLANTIS)) examined whether the positive effects of rt-PA treatment within three hours after the onset of stroke could be repeated even with a treatment within six hours time. However, this question could not be answered affirmatively since no improvement of the clinical symptoms or any decrease in mortality was observed. The higher risk for ICH remained.

Those partially contradictory results have led to a high caution in the use of rt-PA. Already 1996 a publication of the *American Heart Association* pointed out the strong skepticism among doctors with respect to thrombolytic treatment of

stroke; whereas there is no such skepticism with respect to fibrinolytics in the therapy of myocardical infarct (van Gijn J, MD, FRCP, 1996 - Circulation 1996, 93: 1616-1617).

A rationale behind this scepticism was firstly given in a summary of all stroke trials published 1997 (updated in March 2001). According to this review all thrombolytic treatments (urokinase, streptokinase, rt-PA or recombinant urokinase) resulted in a significant higher mortality within the first 10 days after the stroke while the total number of either dead or disabled patients was reduced when the thrombolytics were applied within six hours after stroke onset. This effect was mainly due to ICH. The broad use of thrombolytics for the treatment of stroke was therefore not recommended.

Even before, such results gave reason to some other authors mere sarcastic statement that stroke patients had the choice to either die or to survive disabled (SCRIP 1997: 2265, 26).

Nevertheless, so far the therapy with rt-PA is the only treatment of acute cerebral ischaemia approved by the Food and Drug Administration (FDA) in the USA. However, it is restricted to an application of rt-PA within three hours after the onset of stroke.

The approval of rt-PA was reached in 1996. Before, in the year 1995, first announcements about negative side effects of t-PA became known, which provide an explanatory basis for its dramatic effects when applied in stroke treatment outside the three hour time frame. Accordingly, microglia cells and neuronal cells of the hippocampus produce t-PA which contributes to the glutamate mediated excitotoxicity. This is concluded from a comparative study on t-PA deficient and wild type mice when glutamate agonists were injected in their hippocampus, respectively. The t-PA deficient mice showed a significant higher resistance against external (intrathecal) applied glutamate (Tsirka SE et al., Nature, Vol. 377, 1995, "Excitotoxin-induced neuronal degeneration and seizure

are mediated by tissue plasminogen activator"). These results were confirmed in 1998 when Wang et al. could prove nearly a double quantity of necrotic neuronal tissue in t-PA deficient mice when t-PA was injected intravenously. This negative effect of external t-PA on wild type mice was only approximately 33 % (Wang et al., 1998, Nature, "Tissue plasminogen activator (t-PA) increases neuronal damage after focal cerebral ischaemia in wild type and t-PA deficient mice".)

Further results on the stimulation of excitotoxicity by t-PA were published by Nicole et al. in the beginning of 2001 (Nicole O., Docagne F, Ali C; Margail I; Carmeliet P; MacKenzie ET, Vivien D and Buisson A, 2001: The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling; in: Nat Med 7, 59-64). They could prove that t-PA being released by depolarized cortical neurons could interact with the so called NR1 sub-unit of the glutamate receptor of the NMDA type leading to a cleavage of NR1. This increases the receptor's activity resulting in a higher tissue damage after glutamate agonist NMDA was applied. The NMDA agonist induced excitotoxicity. Thus, t-PA exhibits a neurotoxic effect by activating the glutamate receptor of the NMDA type.

According to a further explanatory concept the neurotoxicity of t-PA results indirectly from the conversion of plasminogen in plasmin. According to this model plasmin is the effector of neurotoxicity (Chen ZL and Strickland S, 1997: Neuronal Death in the hippocampus is promoted by plasmin-catalysed degradation of laminin. Cell: 91, 917-925).

A summarizing outline of the time depending neurotoxic effect of t-PA is given in figure 9. Therein also the increased toxicity of the recombinant t-PA compared to endogenic t-PA becomes evident. This is probably due to rt-PA being able to enter into tissue in higher concentrations.

Despite its neurotoxic side effect and its increasing effect on the mortality t-PA was approved by FDA. This can only be explained by the lack of harmless and

effective alternatives - thus it is due to a very pragmatic cost benefit analysis. Therefore, there is still a need for safe therapies. However, if they were still based on thrombolytica - in case it is not possible to find alternatives to thrombolysis - the problem of neurotoxicity has to be considered (see for example Wang et al. a.a.O.; Lewandowski and Barson 2001 a.a.O.).

Therefore, further examination of known thrombolytica including DSPA (Desmodus rotundus Plasminogen Activator) in order to develop new drugs for stroke was terminated although principally all thrombolytica are potentially suitable. Especially in case of DSPA its potential suitability for this medical indication was pointed out earlier (Medan P; Tatlisumak T; Takano K; Carano RAD; Hadley SJ; Fisher M: Thrombolysis with recombinant Desmodus saliva plasminogen activator (rDSPA) in a rat embolic stroke model; in: Cerebrovasc Dis 1996;6; 175-194 (4th International Symposium on Thrombotic Therapy in Acute Ischaemic Stroke). DSPA is a plasminogen activator with a high homology (resemblance) to t-PA. Therefore - and in addition to the disillusionment resulting from the neurotoxic side effects of t-PA - there were no further expectations, for DSPA being a suitable drug for stroke treatment.

Instead, recent strategies aiming to improve known thrombolytic treatments try to apply the thrombolytic substance no longer intravenously but intraarterially via a catheter directly close to the intravascular thrombus. First experience is available with recombinant produced urokinase. Thus, possibly, the necessary dose for thrombolysis and therewith negative side effects could be reduced. However, this application requires a high technical expenditure and is not available everywhere. Furthermore, the patient has to be prepared in a time consuming action. Time, however, is often limited. Thus, the preparation provides for an additional risk.

Presently, new concepts are directed to anticoagulants such as heparin, aspirin or ancrod, which is the active substance in the poison of the malayan pit viper. Two further clinical trials examining the effects of heparin (*International Stroke Trial* (IST) and *Trial of ORG 10172 in Acute Stroke Treatment* (TOAST)) how-

ever, do not indicate a significant improvement of mortality or a prevention of stroke.

A further new treatment focuses neither on thrombus nor on blood thinning or anti coagulation but attempts to increase the vitality of cells damaged by the interruption of blood supply (WO 01/51613 A1 and WO 01/51614 A1). To achieve this antibiotics from the group of quinons, aminoglycosides or chloramphenicol are applied. For a similar reason it is further suggested to begin with the application of citicholin directly after the onset of stroke. In the body, citicholin is cleaved to cytidine and choline. The cleavage products form part of the neuronal cell membrane and thus support the regeneration of damaged tissue (US 5,827,832).

Recent research on safe treatment is based on the new finding that a part of the fatal consequences of stroke is caused only indirectly by interrupted blood supply but directly to the excito- or neurotoxicity including over activated glutamate receptors. This effect is increased by t-PA (see above). A concept to reduce excitotoxicity is therefore to apply so called neuroprotectives. They can be used separately or in combination with fibrinolytic agents in order to minimize neurotoxic effects. They can lead to a reduced excitotoxicity either directly e.g. as a glutamate receptor antagonist or indirectly by inhibiting voltage dependent sodium or calcium channels (Jin-Mo Lee et al. a.a.O.).

A competitive inhibition (antagonistic action) of the glutamate receptor of NMDA type is possible e.g. with 2-amino-5-phosphonovalerate (APV) or 2-amino-5-phosphonoheptanoate (APH). A non competitive inhibition can be achieved e.g. by substances binding to the phencyclidine side of the channels. Such substances can be phencyclidine, MK-801, dextrorphan or cetamine.

So far, treatments with neuroprotectives have not shown the expected success, possibly because neuroprotectives had to be combined with thrombolytic agents

in order to exhibit their protective effects. This applies to other substances (see also figure 10).

Even a combination of t-PA and neuroprotective agents results only in a limited damage. Nevertheless, the disadvantageous neurotoxicity of the fibrinolytic agent as such is not avoided.

It is therefore an object of the present invention to provide a new therapeutic concept for the treatment of stroke in humans.

According to the invention the use of non-toxic plasminogen activating factors is suggested as outlined in claim 1 for the therapeutic treatment of stroke. Further advantageous uses are subject of independent claims, respectively, as well as of further dependent claims.

The central idea of the invention is the use of a plasminogen activator in the treatment of stroke, of which the mature enzyme exhibits an activity, which is selectively increased by fibrin manifold, namely more than the 650-fold.

The use of the plasminogen activators according to the invention is based on the following findings: Due to tissue damage in the brain caused by stroke the blood brain barrier is damaged or destroyed. Thus, fibrinogen circulating in the blood can enter into the neuronal tissue of the brain. There, it activates t-PA which - indirectly by activating the glutamate receptor or plasminogen - results in further tissue damage. In order to avoid this effect the invention suggests the use of a plasminogen activator which is highly fibrin selective and - as an inversion of the argument - has a reduced potential to be activated by fibrinogen. Thus, this plasminogen activator is not - or compared to t-PA at least substantially less - activated by fibrinogen entering from the blood into neuronal tissue as a result of damaged blood brain barrier, since t-PA's activator fibrin cannot enter the neuronal tissue due to its size. The plasminogen activators according to the invention therefore are non-neurotoxic.

According to a preferred embodiment of the invention, non-toxic plasminogen activators are used, which comprise at least one element of the so called cymogene triade. A comparable triade is known from the catalytic center of serine proteases of the chymotrypsine family consisting of three interacting amino acids aspartate 194, histidine 40 and serine 32. However, this triade does not exist in t-PA which belongs also to the family of chymotrypsine like serine proteases. Nevertheless, it is known, that the directed mutagenesis of native t-PA for the purpose of introducing at least one of the above amino acids at a suitable position results in a reduced activity of the pro-enzyme (single chain t-PA) and to an increased activity of the mature enzyme (double chain t-PA) in the presence of fibrin. Therefore, the introduction of at least one amino acid of the triade - or of an amino acid with the respective function in the triade - can increase the cymogenity of t-PA (i.e. the ratio between the activity of the mature enzyme an the activity of the pro-enzyme). As a result the fibrin specificity is remarkably increased. This is due to conformational interaction between the introduced amino acid residue and/or amino acid residues of the wild type sequence.

It is known that the mutagenesis of the native t-PA with substitution of Phe305 by His (F305H) and of Ala 292 by Ser (A292S) leads to a 20-fold increase of the cymogenity, whereas the variant F305H alone already leads to 5 times higher cymogenity (EL Madison, Kobe A, Gething M-J; Sambrook JF, Goldsmith EJ 1993: Converting Tissue Plasminogen Activator to a Zymogen: A regulatory Triad of Asp-His-Ser; Science: 262, 419-421). In the presence of fibrin these t-PA mutants show an activity increase of 30.000 times (F305H) and 130.000 times (F305H, A292S) respectively. In addition these mutants comprise a substitution of Arg275 to R275E in order to prevent cleavage by plasmin at the cleavage site Aug275-Ile276, thereby converting the single chain t-PA to the double chain form. The mutant site R275E alone leads to a 6.900 fold increase of the fibrin specificity of t-PA (K Tachias, Madison EL 1995: Variants of Tissue-

type Plasminogen Activator Which Display Substantially Enhanced Stimulation by Fibrin, in: Journal of Biological Chemistry 270, 31: 18319-18322).

The positions 305 and 292 of t-PA are homologous to the positions His40 and Ser32 of the known triade of the chymotryptic serine proteases. By the corresponding substitutions introducing histidine or respectively serine, these amino acids can interact with the aspartate477 of t-PA resulting in a functional triade in the t-PA mutants (Madison et al., 1993).

These t-PA mutants can be used for the treatment of stroke according to the invention because they show no or - compared to wild type t-PA - a significantly reduced neurotoxicity due to their increased fibrin specificity. For the purpose of disclosure of the mentioned t-PA mutants F305H; F305H; A292S alone or in combination with R275E we incorporate the publications of Madison et al., (1993) and Tachias and Madison (1995) hereby are fully incorporated by reference.

The increase of fibrin specificity of plasminogen activators can alternatively be achieved by a point mutation of Asp194 (or an aspartate at a homologous position). Plasminogen activators belong to the group of serine proteases of the chymotrypsin family and therefore comprise the conserved amino acid Asp194, which is responsible for the stability of the catalytic active conformation of the mature proteases. It is known that Asp194 interacts with His40 in the cymogenic form of serine proteases. After the cymogene is activated by cleavage this specific interaction is interrupted and the side chain of the Asp194 rotates about 170° in order to form a new salt bridge with Ile16. This salt bridge essentially contributes to the stability of the oxyanion pocket of the catalytic center of the mature serine proteases. It is also present in t-PA.

The introduction of a point mutation replacing Asp194 prima facie impedes the formation or respectively the stability of the catalytic confirmation of serine proteases. Despite this the mutated plasminogen activators show a significant

increase of activity in the presence of their co-factor fibrin - especially in comparison to the mature wild type form - which can only be explained in a way that the interaction with fibrin allows a conformational change promoting catalytic activity (L Strandberg, Madison EL, 1995: Variants of Tissue-type Plasminogen Activator with Substantially Enhanced Response and Selectivity towards Fibrin co-factors, in: Journal of Biological Chemistry 270, 40: 2344-2349).

In conclusion, the Asp194 mutants of the plasminogen activators show a high increase of activity in presence of fibrin which enables their use according to the invention.

In a preferred embodiment according to the invention, a mutant t-PA is used, in which Asp194 is substituted by glutamate (D194E) or respectively by asparagine (D194N). In these mutants the activity of t-PA is reduced 1 to 2000 fold in the absence of fibrin, whereas in the presence of fibrin, an increase of activity by a factor of 498.000 to 1.050.000 can be achieved. These mutants can further comprise a substitution of Arg15 to R15E, which prevents the cleavage of the single chain t-PA at the peptide bond Arg15-Ile16 by plasmin, leading to the double chain form of t-PA. This mutation alone increases the activation of t-PA by fibrin by the factor 12.000. For reasons of disclosure of the t-PA mutations at positions 194 and 15, the publications of Strandberg and Madison (1995) are fully incorporated by reference.

An increase of the fibrin dependency of plasminogen activators can also be achieved by the introduction of point mutations in the so called "autolysis loop". This element is known from trypsin; it can also be found as a homologous part in serine proteases and is especially characterized by three hydrophobic amino acids (Leu, Pro and Phe). The autolysis loop in plasminogen activators is responsible for the interaction with plasminogen. Point mutations in this area can have the effect that the protein-protein interaction between plasminogen and plasminogen activators cannot be effectively formed any longer. These mutations are only functionally relevant in the absence of fibrin. In the presence of

fibrin, they, in contrast, are responsible for an increased activity of the plasminogen activators (K Song-Hua, Tachias K, Lamba D, Bode W, Madison EL, 1997: Identification of a Hydrophobic exosite on Tissue Type Plasminogen Activator That Modulates Specificity for Plasminogen, in: Journal of Biological Chemistry 272; 3, 1811-1816).

In a preferred embodiment t-PA is used showing point mutations in the positions 420 to 423. If these residues are substituted by directed mutagenesis this increases the fibrin dependency of t-PA is increased by a factor up to 61.000 (K Song-Hua et al.). Song-Hua et al. examined the point mutations L420A, L420E, S421G, S421E, P422A, P422G, P422E, F423A and F423E. These publications are fully incorporated by reference for disclosure of the use according to the invention.

According to a further advantageous embodiment a modified tissue plasminogen activator with an amino acid sequence according to SEQ ID No. 1 (figure 13) is used. This modified t-PA differs from the wild type t-PA by the exchange of the hydrophobic amino acids in the position 420 to 423 in the autolysis loop as follows: His420, Asp421, Ala422 and Cys423. This t-PA preferentially contains a phenyl alanine at the position 194. Further the position 275 can be occupied by glutamate. Advantageously the position 194 is occupied by phenyl alanine.

Further, a modified urokinase can be used according to the invention. The urokinase according to the invention can comprise the amino acid sequence according to SEQ ID No. 2 (figure 14) in which the hydrophobic amino acids of the autolysis loop are substituted by Val420, Thr421, Asp422 and Ser423. Advantageously the urokinase is carrying an Ile275 and a Glu194. This mutant shows - in comparison to wild type urokinase - a 500-fold increased fibrin specificity.

Both mutants - urokinase as well as t-PA - were analyzed in semi quantitative tests and showed a increased fibrin specificity in comparison to the wild type t-PA.

The plasminogen activator (DSPA) from the saliva of the vampire bat (*Desmodus rotundus*) also shows a highly increased activity in the presence of fibrin - in specific a 100.000-fold increase. Thus it can be used preferentially according to the invention. The term DSPA comprises four different proteases, which fulfill an essential function for the vampire bat, namely an increased duration of bleeding of the wounds of pray (Cartwright, 1974). These four proteases (DSPA α 1, DSPA α 2, DSPA β , DSPA γ) display a high similarity (homology) to each other and to the human t-PA. They also show similar physiological activities, leading to a common classification under the generic term DSPA. DSPA is disclosed in the patents EP 0 352 119 A1 and of US 6,008,019 and 5,830,849 which are hereby fully incorporated by reference for purpose of disclosure.

DSPA α so far is the best analyzed protease from this group. It has an amino acid sequence with a homology greater than 72 % in comparison to the known human t-PA amino acid sequence (Krättschmar et al, 1991). However, there are two essential differences between t-PA and DSPA. Firstly all DSPA has full protease activity as a single chain molecule, since it is - in contrast to t-PA - not converted into a double chain form (Gardell et al., 1989; Krättschmar et al., 1991). Secondly, the catalytic activity of DSPA is nearly absolutely dependent on fibrin (Gardell et al., 1989; Bringmann et al., 1995; Toschie et al., 1998). For example the activity of DSPA α 1 is increased 100.000 fold in the presence of fibrin whereas the t-PA activity is only increased 550 fold. In contrast, DSPA activity is considerably less strongly induced by fibrinogen, since it only shows a 7 to 9 fold increase (Bringmann et al., 1995). In conclusion, DSPA is considerably more dependent of fibrin and much more fibrin specific as wild type t-PA which is only activated 550-fold by fibrin.

Because of its fibrinolytic characteristics and the strong similarity to t-PA, DSPA is an interesting candidate for the development of a thrombolytic agent. Despite this, the therapeutic use of DSPA as a thrombolytic agent was restricted to the treatment of myocardial infarction in the past, because - due to the contribution of t-PA to the glutamate induced neurotoxicity - no justified hopes existed, that a plasminogen activator which is related to t-PA could reasonably be used for a treatment of acute stroke.

Surprisingly it has been shown that DSPA has no neurotoxic effects even though it shows a high resemblance (homology) to t-PA and even though the physiological effects of the molecules are comparable to a large extent. The above conclusion led to the idea that DSPA after all may be successfully used as a thrombolytic agent for the therapy of stroke without causing severe risks of neuronal tissue damage. Especially interesting is the fact, that DSPA can also be used later than 3 hours after the onset of stroke symptoms.

A further teaching of the present invention that evolved from the above findings is the option to modify or produce further plasminogen activators in such a way that they reveal the essential characteristics of DSPA, especially the lack of the neurotoxicity of t-PA. The basis for this is the investigated relationship between structure and biochemical effects, making it possible to transform neurotoxic plasminogen activators into non-neurotoxic plasminogen activators and thereby to produce non-neurotoxic plasminogen activators on the basis of known or newly discovered neurotoxic plasminogen activators.

The new teaching is based on *in vivo* comparative examinations of the neurodegenerative effect of t-PA on one side and of DSPA on the other side which are performed by using the so called kainic acid model and a model for the examination of NMDA induced lesion of the striatum.

The kainic acid model (also kainic acid injury model) is based on the stimulation of the neurotoxic glutamate cascade by the external application of kainic acid

(KA) as an agonist of the glutamate receptor of the kainic acid type (KA type) and of the NMDA and AMPA glutamate receptors. Using a t-PA deficient mouse stem as an experimental model it was possible to show that the sensitivity of the laboratory animals against kainic acid only reached the level of wild type mice after a supplementary application of external t-PA. In contrast, an infusion of an equimolar concentration of DSPA under the same experimental conditions does not restore the sensitivity to kainic acid (KA). It was concluded that the neurotoxic effect of t-PA was not induced by DSPA. A summary of these results is shown in table 2.

Table 2:

Treatment group	Number of animals	Hippocampal length intact (mm)		Percentage remaining
		Contralateral side mean (SEM)	Ipsilateral side mean (SEM)	
t-PA infusion (1.85 uM) + KA	12	15.99 (0.208)	3.63 (0.458)	22.7*
DSPA infusion (1.85 uM) + KA	11	16.07 (0.124)	13.8 (0.579)	85.87
t-PA infusion (1.85 uM) + PBS	3	16.75 (0.381)	17.08 (0.363)	101.97
DSPA infusion (1.85 uM) + PBS	3	15.75 (0.629)	15.83 (0.363)	100.50
t-PA infusion (0.185 uM) + KA	3	15.60 (0.702)	5.07 (1.09)	32.5
DSPA infusion (18.5 uM) + KA	3	16.06 (0.176)	13.80 (1.22)	85.93

* P < 0.0001

Quantitative examinations based on this model revealed that even a 10-fold increase of the DSPA concentration could not restore the sensitivity of the t-PA deficient mice to the KA treatment whereas already a 10-fold lower t-PA concentration led to KA induced tissue damages. This leads to the conclusion that DSPA possesses an at least 100 fold lower neurotoxic potential as t-PA with

respect to the stimulation of the neurodegeneration after KA treatment (see also figures 11 and 12).

In the second model of neurodegeneration, the possible effects of t-PA as well as DSPA on the stimulation of the NMDA dependent neurodegeneration were compared to wild type mice. For this purpose, NMDA (as an agonist of the glutamate receptor of the NMDA type) was injected in wild type mice alone or in combination with either t-PA or DSPA. This model allows the comparison of the effects of these proteases under conditions, which always lead to a neurodegeneration and to an influx of plasma proteins due to the break down of the blood brain barrier (Chen et al., 1999).

While working on this model the injection of NMDA led to reproducible lesions in the striatum of mice. The volume of lesions was increased by a combined injection of t-PA and NMDA by at least 50 %. The co-injection with DSPA α 1 in contrast did not lead to an increase or extension of the lesions caused by NMDA. Even in the presence of plasma proteins which can freely diffuse in the region of the lesion induced by NMDA, DSPA did not result in an increase neurodegeneration (see also table 3).

Table 3:

Treatment group	Number of wild-type mice	Mean lesion volume (mm ³) (SEM)
NMDA alone	8	1.85 (0.246)
NMDA + t-PA	8	3.987 (0.293) *
NMDA + DSPA	8	1.656 (0.094) **
t-PA alone	3	0.20 (0.011)
DSPA alone	3	0.185 (0.016)

** Not significant

} * P < 0.0001

These results show that fibrin-free DSPA - in contrast to t-PA - behaves like an almost inert protease in the central nervous system of a mammal and also of a human - and therefore does not contribute to the neurotoxic effects caused by KA or NMDA. Despite of the prejudice against the therapeutic use of t-PA like proteins in stroke, this lacking neurotoxicity makes DSPA a suitable thrombolytic agent for the treatment of acute stroke.

First results of the clinical trials show the transferability of these results also for the treatment of stroke in humans. It was found that significant improvements can be achieved in patients after a successful perfusion (improvement by 8 points NIHSS or NIHSS score 0 to 1). Table 1 shows the data.

Table 1:

Patient		NIHSS				
	Baseline	Post Tmt	Day 7	Day 30	Day 90	sAEs
1001	12	7	4	4	*	Re-Infarction
1002	8	9	2	0	0	
1003	8	10	12	10	*	
1004	8	4	2	0	0	
1005	11	11	4	5	*	
1006	9	7	1	*	*	
1007	14	6	*	*	*	
2001	19	20	--	--	--	ICH, death
2002	15	21	--	--	--	ICH, death
3001	8	7	6	5	*	
3002	15	16	9	8	*	
3003	10	19	21	*	--	Death day 39

The lacking neurotoxicity of DSPA and of the other non-neurotoxic plasminogen activators (see above) offer the special advantage in stroke treatment that the

use of these plasminogen activators - in contrast to the wild type t-PA - is not limited to a short maximum period of only 3 hours after the onset of stroke. In contrary, the treatment can be initiated later - for example after 6 hours or even later, since there is nearly no risk of stimulating excitotoxic responses. First clinical trials with DSPA prove a safe treatment of patients even in a time range of over 6 to 9 hours after the onset of stroke symptoms.

This option of a timely unlimited treatment with non-neurotoxic activators is of special importance, since it allows for the first time to treat patients with acute stroke symptoms safely even when diagnosis is delayed or the onset of the stroke cannot be determined with sufficient security. In the prior art, this group of patients was excluded from thrombolytic therapy with plasminogen activators due to unfavorable risk estimation. Consequently, an essential contra-indication for the authorized use of a thrombolytic agent for stroke is eliminated.

DSPA as well as further non-neurotoxic plasminogen activators show no tissue damaging side effects. However, it can be advantageous to apply them in combination with a neuroprotective agent for the treatment of stroke in order to limit the tissue damages induced by the glutamate occurring naturally in the human body. Neuroprotective agents inhibiting the glutamate receptor competitively or non-competitively can be used. Useful combinations are e. g. with the known inhibitors of the glutamate receptors of the NMDA type, the kainic acid type or the quisqualate type, as for example APV, APH, phencyclidine, MK-801, dextrorphan or cetamine.

Further a combination with cations can be advantageous since cations, especially Zn-ions, block the cation channel regulated by the glutamate receptor and can therefore reduce neurotoxic effects.

In a further advantageous embodiment, non-neurotoxic plasminogen activators can be combined with at least one further therapeutic agent or with a pharmaceutically tolerable carrier. The combination with a therapeutic agent which

supports the reduction of tissue damage by vitalizing the cells is especially advantageous, since it contributes to the regeneration of already damaged tissue or serves for the prevention of further stroke incidents. Advantageous examples are combinations with antibiotics as quinones, anticoagulants as heparin or hirudin as well as with citicholine or acetylsalicylic acid.

A combination with at least one thrombin inhibitor can also be advantageous. Preferentially, thrombomodulin and thrombomodulin analogs like for example solulin, triabin or pallidipin can be used. Further combinations with anti-inflammatory substances are advantageous, since they influence the infiltration by leucocytes.

Comparing examinations of t-PA and DSPA are methods:

1. Animals

Wild-type mice (c57/Black 6) and t-PA deficient mice (t-PA^{-/-}-mice) (c57/Black 6) (Carmeliet et al., 1994) were supplied by Dr. Peter Carmeliet, Leuven, Belgium.

2. Protein extraction from brain tissue

The assessment of proteolytic activity in brain tissue following infusion of either t-PA or DSPA α 1 was performed by zymographic analysis (Granelli-Piperno and Reich, 1974). After an infusion over a period of seven days into the hippocampus, mice were anaesthetised, then transcardially perfused with PBS and the brains removed. The hippocampus region was removed, transferred to eppendorf tubes and incubated in an equal volume (w/v) (approx. 30-50 μ m) of 0,5 % NP-40 lysis buffer containing no protease inhibitors (0,5 % NP-40, 10 mM Tris-HCl pH 7,4, 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA). The brain extracts were homogenized by means of a hand-held glass homogeniser and left on ice for 30 minutes. The samples were then centrifuged and the supernatant was removed. The amount of proteins present was determined (Bio-Rad-reagent).

3. Zymographic analysis of the proteases

The proteolytic activity in the samples and the brain tissue extracts was determined by zymographic analysis according to the method of Granelli, Piperno and Reich (1974). The samples with recombinant proteins (up to 100 nM) or the brain tissue extracts (20 µg) were subjected to a (10 %) SDS-PAGE under non-reducing conditions. The gels were removed from the plates, washed in 1 % triton X 100 for 2 hours and then overlaid onto an agarose gel containing polymerized fibrinogen and plasminogen (Granelli, Piperno and Reich, 1974). The gels were incubated at 37°C in a humidified chamber until proteolysed zones appeared.

4. Intra-hippocampal infusion of t-PA, DSPA and subsequent injection of kainic acid

The kainic acid injury model was based on studies of Tsirka et al. (1995). The animals were injected intraperitoneally (i. p.) with atropine (4 mg/kg) and then anaesthetised with an i. p. injection of sodium pentobarbitol (70 mg/kg). Afterwards mice were placed in a stereotaxic frame and a micro-osmotic pump (Alzet model 1007D, Alzet CA. USA) containing 100 µl of either PBS or recombinant human t-PA (0,12 mg/ml, 1,85 µM) or DSPA α 1 (1,85 µM) was implanted subcutaneously between the shoulder blades. The pumps were connected via sterile tubes to a brain cannula and inserted through a burr opening made through the skull at coordinates bregma -2,5 mm, midiolateral 0,5 mm and dorsoventral 1,6 mm in order to introduce the liquid near the midline. The cannula was fixed at the desired position and the pumps were allowed to infuse the respective solutions at a rate of 0,5 µl per hour for a total of 7 days.

Two days after infusion of the proteases the mice were reanaesthetised and again placed in the stereotaxic frame. Afterwards 1,5 nmol of kainic acid (KA) in 0,3 µl PBS was injected unilaterally into the hippocampus. The coordinates were:

bregma -2,5 mm, medial-lateral 1,7 mm and dorsoventral 1,6 mm. The excitotoxin (KA) was delivered for a duration of 30 seconds. After the kainic acid treatment the injection needle remained at these coordinates for further 2 minutes in order to prevent a reflux of the liquid.

5. Brain processing procedure

Five days after KA injection, the animals were anaesthetised and transcardially perfused with 30 ml PBS followed by 70 ml of a 4 % paraformaldehyd solution, post fixed in the same fixative followed by incubation in 30 % sucrose for further 24 hours. Coronal sections (40 μ m) of the brain were then cut on a freezing microtome and either counter-stained with thionin (BDH, Australia) or processed for immunohistochemical examination as described below.

6. Quantification of neuronal loss within the hippocampus

The quantification of neuronal loss in the CA1-CA3 hippocampal subfields was performed as previously described (Tsirka et al., 1995; Tsirka et al., 1996). Five consecutive parts of the dorsal hippocampus from all treatment groups were prepared taking care that the parts indeed comprised the place of the CA-injection and lesion area. The hippocampal subfields (CA1-CA3) of these sections were traced by means of camera lucida drawings of the hippocampus. The entire lengths of the subfields was measured by comparison to 1 mm standards traced under the same magnification. The lengths of tissue with viable pyramidal neurons (having normal morphology) and lengths of tissue devoid of neurons (no cells present, no thionin staining) was determined. The lengths, representing intact neurons and neuronal losses over each hippocampal subfield were averaged across sections and the standard deviations were determined.

7. Intra-striatal NMDA excitotoxic lesions with or without t-PA or DSPA

Wild type mice (c57/Black 6) were anaesthetised and placed in a stereotaxic frame (see above). Mice then received an unilateral injection of 50 nmol NMDA in the left striatum, injected alone or in combination with either 46 μ M rt-PA or 46 μ M DSPA α 1. As controls t-PA and DSPA were also injected alone (both at a concentration of 46 μ M). The injection coordinates were: bregma -0,4 mm, midiolateral 2,0 mm and dorsoventral 2,5 mm. The solutions (1 μ l total volume for all treatments) were transferred over a period of 5 minutes at a rate of 0,2 μ l/min and the needle was left in place for further 2 minutes after the injection in order to minimize the reflux of fluid. After 24 hours the mice were anaesthetised and perfused transcardially with 30 ml PBS followed by 70 ml of a 4 % paraformaldehyde solution, post fixed in the same fixative for 24 hours with followed by incubation in 30 % sucrose for further 24 hours. Brains were then cut (40 μ m) on a freezing microtome and mounted onto gelatin coated glass slides.

8. Quantification of the lesion volume following NMDA injection

The quantification of the striatal lesion volume was performed using the method described by Callaway et al. (2000). Ten consecutive coronal sections spanning the lesioned area were prepared. The lesioned area was visualised using the Callaway method and the lesion volume was quantified by the use of a micro computer imaging device (MCID, Imaging Research Inc., Brock University, Ontario, Canada).

9. Immunohistochemistry

Immunohistochemistry was performed using standard methodologies. Coronal sections were immersed in a solution of 3 % H₂O₂ and 10 % methanol for 5 minutes followed by an incubation in 5 % normal goat serum for 60 minutes. The sections were incubated over night either with an anti-GFAP antibody (1:1.000; Dako, Carpinteria, CA, USA) for the detection of astrocytes, with an anti-MAC-1 antibody (1:1.000; Serotec, Raleigh, NC, USA) for the detection of microglia or with polyclonal anti-DSPA antibodies (Schering AG, Berlin). After rinsing, the

sections were incubated with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). This was followed by a final incubation with avidin/biotin-complex (Vector Laboratories, Burlingame, CA, USA) for 60 minutes before visualisation with 3,3'-diaminebebcidine/0,03 % H₂O₂. Sections were then mounted on gelatin coated slides, dried, dehydrated and coverslipped with permount.

B. Results

1. Infusion of t-PA or DSPA disperses into the hippocampus of t-PA ^{-/-} mice and retains proteolytic activity

The initial experiments were designed to confirm that both DSPA and t-PA retain their proteolytic activity for the 7 day period of the infusion. To this end, aliquots of t-PA and DSPA (100 nmol) were incubated at 37 °C and at 30°C for 7 days in a water bath. In order to determine the proteolytic activity, 5 fold serial dilutions of the probes were subjected to SPS-PAGE under non-reducing conditions and proteolytic activity was assessed by zymographic analyses. An aliquot of t-PA and DSPA which had been kept frozen for a period of 7 days was used as a control. As can be seen in figure 1 there was only a minor loss of DSPA or t-PA activity at an incubation with either 30°C or 37°C over this period of time.

2. t-PA and DSPA activity is recovered in hippocampal extracts prepared from t-PA ^{-/-} mice following infusion

First it had to be confirmed that the infused proteases were present in the brain of the infused animals and also retained their proteolytic activity while being in this compartment. To address this point, t-PA ^{-/-} were infused for seven days with either t-PA or DSPA (see above). Mice were then transcardially perfused with PBS and the brains removed. The ipsilateral and contralateral hippocampal regions were isolated as well as a region of the cerebellum (taken as a negative control). Tissue samples (20 µg) were subjected to SDS-PAGE and zymographic

analysis according to the description in the methods section. As can be seen in figure 2, both t-PA and DSPA activities were detected in the ipsilateral region of the hippocampus, while some activity was also detected on the contralateral side. This indicates that the infused proteases not only retained their activity in the brain but had also diffused within the hippocampal region. As a control, no activity could be detected in the extract prepared from the cerebellum.

3. Immunohistochemical assessment of DSPA

To further confirm that DSPA had indeed diffused into the hippocampal region, coronal brain sections of t-PA $-/-$ mice were analysed immunohistochemically after DSPA infusion. DSPA-antigen was detected in the hippocampal region with the most prominent staining in the area of the infusion site. This result confirms that the infused DSPA is soluble and is indeed present in the hippocampus.

4. DSPA infusion does not restore kainic-acid mediated neurodegeneration *in vivo*

t-PA $-/-$ mice are characteristically resistant to kainic acid (KA) mediated neurodegeneration. However, intrahippocampal infusion of rt-PA completely restores the sensitivity to KA-mediated injury. To determine whether DSPA could be substituted for t-PA in this model, t-PA $-/-$ mice were infused intrahippocampally with either t-PA or DSPA using a mini-osmotic pump. For both groups 12 mice were tested. 2 days later the animals were injected with kainic acid and left to recover. 5 days later the animals were killed and the brains removed and prepared (see above). As controls, t-PA $-/-$ mice were also infused with PBS prior to KA treatment (N = 3).

Coronal brain sections were prepared and the neurons detected by Nissl staining. As shown in figure 4, t-PA $-/-$ mice infused with PBS were resistant to subsequent challenge with KA. However, infusion of recombinant t-PA restored sensitivity to KA treatment. In contrast, infusion of the same concentration of

DSPA into the hippocampal region did not alter the sensitivity of the animals to KA.

A quantitation of those results was based on data obtained from 12 mice in each group. In 2 of the 12 mice infused with DSPA a small extend of neurodegeneration was observed. The reason for that is unclear and possibly not related to the presence of DSPA. The combined data consider this minor effect that was observed in the case of these 2 animals. All 12 mice treated with t-PA were sensitive against the KA treatment. These results show that in case of an infusion of t-PA or DSPA α 1 in equimolar concentrations only the administering of t-PA led to the restoration of sensitivity to KA induced neurodegeneration.

5. DSPA infusion does not result in microglial activation

The restauration of the KA sensitivity of the t-PA $-/-$ mice caused by a t-PA infusion also results in a microglia activation (Rogove et al., 1999). To assess the degree of microglial activation following t-PA or DSPA infusion and subsequent KA treatment, coronal sections of mice were subjected to an immunohistochemical staining for activated microglia cells using the Mac-1 antibody. The restauration of KA sensitivity following t-PA infusion resulted in a clear increase in Mac-1 positive cells. This was not observed in mice infused with DSPA. Hence, the presence of DSPA does not result in the activation of microglia cells following KA treatment.

6. Titration of DSPA and t-PA in the mice hippocampus region.

The concentration of t-PA used for the infusion was based on the concentration described by Tsirka et al. (1995) (100 μ l of 0,12 mg/ml [1,85 μ M]). The KA-injury experiments were repeated using a 10-fold lower of t-PA (0,185 μ M) and a 10-fold higher amount of DSPA (18,5 μ M). The lower t-PA concentration was still able to restore the sensitivity to KA treatment (n=3). Of special interest was the finding that the infusion of 10 fold increased DSPA concentration only caused a

little neuronal loss following KA treatment. These data strongly point out that DSPA does not lead to an increase of sensitivity to KA.

7. Effect of t-PA and DSPA on NMDA-dependent neurodegeneration in wild type mice

The effects of t-PA and DSPA were also examined in a model of neurodegeneration in wild type mice. The injection of t-PA in the striatum of these mice provably led to an increase of the neurodegenerative effects caused by the glutamate analogue NMDA (Nicole et al., 2001).

NMDA was injected into the striatal region of wild type mice in the presence of t-PA or DSPA (each 46 μ M) with a total volume of 1 μ l. After 24 hours the brains were removed and the size of the lesions was quantified according to the Callaway method (Callaway et al., 2000) (see above). As can be seen in figure 7, injection of NMDA alone caused a reproducible lesion in all treated mice (N=4). When t-PA and NMDA were applied together, the size of the lesions was increased about 50 % ($P < 0,01$, $n=4$). In a clear contrast the co-injection of NMDA and the same concentration of DSPA did not lead to an increase in lesion size compared to NMDA alone.

Injection of t-PA or DSPA alone did not lead to a detectable neurodegeneration. The lacking effect of t-PA when being administered alone is consistent with the results of Nicole et al. (2001). These data show that the presence of DSPA does not increase neurodegeneration even during a neurodegenerative event.

In order to confirm that the injection of DSPA had indeed spread into the hippocampal region, immunohistochemistry was performed on coronal sections by use of the DSPA antibody. The examination showed that DSPA did indeed enter the striatal region.

Kinetic analysis of the plasminogen activation by indirect chromogen test

Indirect chromogen tests of the t-PA activity were performed using the substrate Lys-plasminogen (American Diagnostica) and spectrozyme PL (American Diagnostica) according to Madisan E.L., Goldsmith E.J., Gerard R.D., Gething M.-J., Sambrook J.F. (1989) Nature 339 721-724; Madison E.L.O., Goldsmith E.J., Gething M.J., Sambrook J.F. and Bassel-Duby R.S. (1990) Proc. Natl. Acad. Sci U.S.A 87, 3530-3533 as well as Madison E.L., Goldsmith E.J., Gething M.J., Sambrook J.F. and Gerard R.D. (1990) J. Biol. Chem 265, 21423-21426. Tests were performed both in the presence and absence of the co-factor DESAFIB (American Diagnostica). DESAFIB is a preparation of soluble fibrin monomeres gained by the cleavage of highly pure human fibrinogen with the protease batroxobin. Batroxobin cleaves the Arg¹⁶-Gly¹⁷-binding in the A α -chain of fibrinogen and thereby releases fibrinopeptid A. The resulting des-AA-fibrinogen representing fibrin I monomers is soluble in the absence of the peptide Gly-Pro-Arg-Pro. The concentration of Lys-plasminogen was varied from 0,0125 up to 0,2 μ M in the presence of DESAFIB and from 0,9 to 16 μ M in absence of the co-factor.

Indirect chromogen tests in the presence of different stimuli.

Indirect chromogen standard tests were performed according to the publications cited above. Probes of 100 μ l total volume containing 0,25-1 ng enzyme, 0,2 μ M Lys-plasminogen and 0,62 mM spectrozyme PL were used. The tests were performed either in the presence of buffer, 25 μ g/ml DESAFIB, 100 μ g/ml cyanogen bromide fragments of fibrinogen (American Diagnostica) or 100 μ g/ml of the stimulatory 13 amino acid peptide P368. The analysis were performed in microtiter-plates and the optic density was determined at a wave length of 405 nm every 30 seconds for 1 hour in a "Molecular Devices Thermomax". The reaction temperature was 37 °C.

Claims:

1. Use of a plasminogen activating factor for the treatment of stroke wherein the activity of the plasminogen activating factor is enhanced by more than 650 fold in the presence of fibrin.
2. Use of a plasminogen activating factor according to claim 1 wherein the plasminogen activating factor comprises at least a histidine or serine residue forming together with an aspartate residue at least a part of a cymogene triade.
3. Use of a plasminogen activating factor according to claim 2 wherein the serine residue is located at a position at least partly homologous to position 292, the histidine residue is located at a position at least partly homologous to position 305 and the aspartate residue is located at a position at least partly homologous to position 447 of t-PA.
4. Use of a plasminogen activating factor according to claim 3 wherein the plasminogen activating factor is selected from the group of the following t-PA mutants: t-PA/R275E; t-PA/R275E, F305H; t-PA/R275E, F305H, A292S.
5. Use of a plasminogen activating factor according to claim 1 wherein the plasminogen activating factor carries a point mutation of the Asp194 or of an aspartate in a homologue position, leading to a reduced stability of the catalytically active conformation of the plasminogen activating factor in the absence of fibrin.
6. Use according to claim 5 wherein Asp194 is substituted by glutamate or asparagine.
7. Use according to claim 6 wherein t-PA carries a substitution of Asp194 to Glu194 or Asn194.

8. Use of a plasminogen activating factor according to claim 1 wherein the plasminogen activating factor comprises at least one mutation in its autolysis loop, which reduces the functional interactions between plasminogen and plasminogen activating factor in the absence of fibrin.
9. Use according to claim 8 wherein at least one mutation in the autolysis loop affects the amino acid positions 420 to 423 of wild type t-PA or homologous positions.
10. Use according to claim 9 wherein the mutation is selected from the group consisting of the following mutants: L420A, L420E, S421G, S421E, P422A, P422G, P422E, F423A and F423E.
11. Use of a plasminogen activating factor according to claim 1 wherein the plasminogen activating factor is a cymogene comprising at least one point mutation preventing the catalysis by plasmin.
12. Use of a plasminogen activating factor according to claim 11 wherein the point mutation is located at the position 15 or 275 of t-PA or at a position homologous to that.
13. Use according to claim 12 wherein glutamate is in the position 15 or 275.
14. Use of a plasminogen activating factor according to claim 1 wherein the plasminogen activating factor was isolated from the saliva of the vampire bat (DSPA).
15. Use of a plasminogen activating factor according to at least one of the above claims for the therapeutic treatment of stroke in humans at least 3 hours after stroke onset.

16. Use of a plasminogen activating factor according to at least one of the above claims for the therapeutic treatment of stroke in humans at least 6 hours after stroke onset.
17. Use of a plasminogen activating factor according to at least one of the above claims for the therapeutic treatment of stroke in humans at least 9 hours after stroke onset.
18. Use of a plasminogen activating factor according to at least one of the above claims for the therapeutic treatment of stroke patients in which the onset of stroke is temporally not exactly determined.
19. Use of a plasminogen activating factor according to at least one of the above claims for the therapy of stroke under avoidance of the neurotoxicity of wild type t-PA.
20. Tissue plasminogen activating factor with an autolysis loop comprising His420, Asn421, Ala422 and Cys423.
21. Tissue plasminogen activating factor according to claim 20 characterized in a point mutation in position 194, which reduces the stability of the catalytically active conformation of the plasminogen activating factor in the absence of fibrin.
22. Tissue plasminogen activating factor according to claim 21 characterized in Phe194.
23. Tissue plasminogen activating factor according to at least one of the claim 20 to 22 characterized in at least one point mutation which prevents the catalysis by plasmin.

24. Tissue plasminogen activating factor according to claim 23 characterized in Glu275.
25. Tissue plasminogen activating factor with an amino acid sequence according to Seq. ID No. 1.
26. Urokinase with an autolysis loop comprising Val420, Thr421, Asp422 and Ser423.
27. Urokinase according to claim 26 characterized in a point mutation in position 194, which reduces the stability of the catalytic active conformation of the urokinase in absence of fibrin.
28. Urokinase according to claim 27 characterized in Glu194.
29. Urokinase according to at least one of the claims 26 to 28 characterized in at least one point mutation which prevents the catalysis by plasmin.
30. Urokinase according to claim 29 characterized in Ile275.
31. Urokinase with an amino acid sequence according to Seq. ID No. 2.
32. Pharmaceutical composition containing a plasminogen activating factor according to at least one of the above claims and at least one additional pharmaceutically active component or its pharmaceutically acceptable salt.
33. Pharmaceutical composition according to claim 32 characterized in a neuroprotective agent.
34. Pharmaceutical composition according to claim 33 characterized in a glutamate receptor antagonist.

35. Pharmaceutical composition according to claim 34 characterized in a competitive or non-competitive antagonist.
36. Pharmaceutical composition according to claim 33 characterized in at least one thrombin inhibitor, preferentially selected from the group of the following substances: thrombomodulin, thrombomodulin analogues, triabin, pallidipin or solulin.
37. Pharmaceutical composition according to claim 33 characterized in at least one anticoagulant agent, preferentially selected from the group of the following anticoagulant agents: hirudin, heparin, acetylsalicylic acid or ancrod.
38. Pharmaceutical composition according to claim 33 characterized in anti-inflammatory substances.
39. Pharmaceutical compositions according to claim 33 characterized in antibiotic agent.
40. Pharmaceutical composition according to claim 33 characterized in citicholine.
41. Use of a pharmaceutical composition according to at least one of the claims 36 to 45 according to at least one of the claims 1 to 19.
42. Method for the production of a drug for the treatment of stroke containing one non-neurotoxic plasminogen activating factor comprising at least one of the following steps for the modification of the plasminogen activating factor:
 - introduction of at least a part of a cymogenic triade;
 - substitution of Asp194 or a homologous aspartate for reducing the stabilisation of the catalytic active conformation in the absence of fibrin;

- substitution of the hydrophobic amino acid residues in the autolysis loop or in homologous peptide sections to that;
- introduction of a mutation in the cymogene for preventing the catalysis of the cymogene by plasmin.

43. Drug, produced according to claim 42.

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