

Theme Issue Article

Thrombolytic agents

Désiré Collen, H. Roger Lijnen

Center for Molecular and Vascular Biology, KU Leuven, Belgium

Summary

Thrombolytic agents are plasminogen activators that convert the zymogen plasminogen to the active enzyme plasmin, which degrades fibrin. Elucidation of the molecular mechanism of physiological fibrinolysis opened up a new era of fibrin-specific thrombolysis. Fibrin-specific plasminogen activators, including tissue-type plasminogen activator (t-PA), single-chain urokinase-type plasminogen activator (scu-PA) and staphylokinase (Sak),

preferentially activate fibrin-associated plasminogen. Generated plasmin remains associated with fibrin, where it is protected from rapid inhibition and can efficiently degrade fibrin, avoiding systemic activation of the fibrinolytic system. Following a decade of clinical investigation t-PA and variants thereof are routinely used for treatment of patients with thromboembolic disease.

Keywords

Thrombolysis, plasminogen activator, thromboembolic disease

Thromb Haemost 2005; 93: 627-30

Introduction

Thrombolysis consists of the pharmacological dissolution of a blood clot by administration of thrombolytic agents that activate the fibrinolytic system. Thrombolytic agents are plasminogen activators which convert the proenzyme, plasminogen, into an active enzyme, plasmin, which digests fibrin to soluble degradation products. Inhibition of the fibrinolytic system occurs at the level of the plasminogen activator (mainly by plasminogen activator inhibitor-1, PAI-1) and at the level of plasmin (mainly by α_2 -antiplasmin).

As early as 1933, Tillett and Garner reported a streptococcal substance, streptokinase, that could induce fibrinolysis via activation of plasminogen (1). In 1947, Astrup and Permin showed that animal tissues contain an agent that activates plasminogen (2). This factor, originally termed fibrinokinase, was later also identified in human blood and is now called tissue-type plasminogen activator (t-PA). In 1948 staphylokinase (Sak), secreted by certain strains of *Staphylococcus aureus*, was shown to have profibrinolytic properties (3). Macfarlane and Pilling (4) found fibrinolytic activity in urine and Williams (5) demonstrated that this was due to the presence of a plasminogen activator, now called urokinase (urokinase-type plasminogen activator, u-PA). In the 1970s, Bernik (6) and Nolan et al. (7) observed latent urokinase-like activity in conditioned media of some human cells, now referred to as single-chain u-PA (scu-PA or pro-urokinase).

Identification of most components of the fibrinolytic system and detailed biochemical studies of their interactions have re-

sulted in elucidation of the molecular mechanism of fibrin-specific physiological fibrinolysis (8). These studies provided the conceptual basis to classify plasminogen activators into fibrin-selective and non fibrin-selective agents. The latter, including streptokinase and two-chain u-PA (tcu-PA), activate both plasminogen in the circulating blood and fibrin-bound plasminogen. Generated plasmin is rapidly inactivated by α_2 -antiplasmin and will, after saturation of the inhibitor, degrade other plasma proteins causing the so-called "lytic state". In contrast, fibrin-selective agents, including t-PA, scu-PA and Sak, preferentially activate fibrin-bound plasminogen. Generated plasmin remains associated with fibrin and is protected from rapid inhibition by α_2 -antiplasmin, because the structures required for this inhibition (active site and lysine binding sites of plasmin) are not available to the inhibitor. This mechanism has triggered interest in using such fibrin-specific agents for thrombolytic therapy. Their main properties are summarized below.

Tissue-type plasminogen activator

A historical perspective on the production of t-PA and its development for thrombolytic therapy has recently been published (9). Wild-type recombinant t-PA (rt-PA, alteplase) was first obtained as a single-chain serine proteinase of 70 kDa, consisting of 527 amino acids with Ser as the NH₂-terminal amino acid; native t-PA actually contains an NH₂-terminal extension of three amino acids, but in general the initial numbering system has been maintained. Limited plasmic hydrolysis of the Arg²⁷⁵-Ile²⁷⁶ peptide

Correspondence to:

Désiré Collen

Center for Molecular and Vascular Biology

KU Leuven, Campus Gasthuisberg, O & N

Herestraat 49, B-3000 Leuven

Belgium

Tel.: + 32-16-345772, Fax: + 32-16-345990

E-mail: desire.collen@med.kuleuven.ac.be

Received November 8, 2004

Accepted December 16, 2004

Prepublished online March 7, 2005 DOI: 10.1160/TH04-11-0724

bond converts t-PA to a two-chain molecule held together by one interchain disulfide bond. The t-PA molecule contains four domains: 1) an NH₂-terminal region of 47-residues (residues 4 to 50) which is homologous with the finger domains of fibronectin; 2) residues 50 to 87 which are homologous with epidermal growth factor; 3) two kringle regions comprising residues 87 to 176 and 176 to 262 which are homologous with the five kringles of plasminogen; and 4) a serine proteinase domain (residues 276 to 527) with the active site residues His³²², Asp³⁷¹ and Ser⁴⁷⁸. There are three potential N-glycosylation sites, at Asn¹¹⁷, Asn¹⁸⁴ and Asn⁴⁴⁸ (10). In contrast to the single-chain precursor of most serine proteinases, single-chain t-PA is enzymatically active. The normal plasma concentration of endogenous t-PA is about 5–10 ng/ml, but varies strongly under different physiological and pathological conditions (11). Alteplase is produced by expression in Chinese hamster ovary cells (Activase[®], Actilyse[®]).

Structure-function analysis has revealed specific functions for the t-PA domains. Thus, high-affinity binding of t-PA to fibrin is mediated by the finger and kringle 2 domains; stimulation of its activity by fibrin also involves the finger, (kringle1?) and kringle 2 domains; *in vivo* clearance is regulated by the finger and growth factor domains and by the carbohydrate side chains; rapid inhibition by PAI-1 requires interaction with a positively charged amino acid sequence; and the enzymatic activity resides in the serine proteinase domain (11).

t-PA is a poor enzyme in the absence of fibrin, but the presence of fibrin strikingly enhances the activation rate of plasminogen (12). During fibrinolysis, fibrinogen and fibrin are continuously modified by cleavage with thrombin or plasmin, yielding a diversity of reaction products (13). Optimal stimulation of t-PA is only obtained after early plasmin-cleavage in the COOH-terminal A α -chain and the NH₂-terminal B β -chain of fibrin, yielding fragment X-polymer. Kinetic data support a mechanism in which fibrin provides a surface to which t-PA and plasminogen adsorb in a sequential and ordered way yielding a cyclic ternary complex (12). Formation of this complex results in an enhanced affinity of t-PA for plasminogen, yielding up to three orders of magnitude higher catalytic efficiencies for plasminogen activation. This is mediated at least in part by COOH-terminal lysine residues generated by plasmin cleavage of fibrin. Plasmin formed at the fibrin surface has both its lysine binding sites and active site occupied and is thus only slowly inactivated by α_2 -antiplasmin (half-life of about 10–100 s, as compared to about 0.1 s for free plasmin) (8). These molecular interactions mediate the fibrin-specificity of t-PA, triggering its clinical use.

Numerous studies have compared the thrombolytic properties of alteplase with those of other agents, culminating in the GUSTO trial and its angiographic substudy (14, 15), which conclusively established the potential of rt-PA for thrombolytic therapy in patients with acute myocardial infarction (AMI). The rapid *in vivo* clearance of rt-PA (half-life of about 6 min in man), necessitating the use of large therapeutic doses, and the occurrence of bleeding and reocclusion has, however, triggered the search for improved rt-PA moieties. By deletion or substitution of functional domains, by site-specific point mutations and/or by altering the carbohydrate composition, mutants of rt-PA have

been produced with higher fibrin-specificity, more zymogenicity, slower clearance from the circulation and resistance to plasma proteinase inhibitors.

Retepase (Rapilysin[®] or Ekokinase[®]) is a single-chain non glycosylated deletion variant of rt-PA consisting only of the kringle 2 and the proteinase domain; it contains amino acids 1–3 and 176–527 (deletion of Val⁴-Glu¹⁷⁵); the Arg²⁷⁵-Ile²⁷⁶ plasmin cleave site is maintained. It has a similar plasminogenolytic activity as wild-type rt-PA in the absence of a stimulator, but its activity in the presence of a stimulator is 4-fold lower, and its binding to fibrin is 5-fold lower. Retepase and rt-PA are inhibited by PAI-1 to a similar degree (16). Retepase has an initial half-life of 14–18 min in patients with AMI; reteplase and alteplase appear to have similar benefits in terms of 30 day mortality and frequency of hemorrhagic stroke (GUSTO-III trial) (17). In tenecteplase (TNK-rt-PA), replacement of Asn¹¹⁷ with Gln (N117Q) deletes the glycosylation site in kringle 1, whereas substitution of Thr¹⁰³ by Asn (T103N) reintroduces a glycosylation site in kringle 1, but at a different locus; these modifications substantially decrease the plasma clearance rate (half-life of 17–20 min). In addition, the amino acids Lys²⁹⁶-His²⁹⁷-Arg²⁹⁸-Arg²⁹⁹ are each replaced with Ala, which confers resistance to inhibition by PAI-1 (18). Tenecteplase has a similar ability as wild-type rt-PA to bind to fibrin, and lyses fibrin clots in a plasma milieu with enhanced fibrin-specificity and delayed inhibition by PAI-1 (18). Its thrombolytic properties in patients with AMI have been reviewed in detail (19). Lanoteplase is a deletion mutant of rt-PA (without the finger and growth factor domains) in which glycosylation at Asn¹¹⁷ is lacking (20). Montepase has a single amino acid substitution in the growth factor domain (Cys⁸⁴ to Ser) (21). Pamiteplase has deletion of the kringle 1 domain and substitution of Arg²⁷⁵ to Glu (rendering it resistant to conversion to a two-chain molecule by plasmin) (22).

Different molecular forms of the Desmodus salivary plasminogen activator (DSPA) have been characterized. Two high M_r forms, DSPA α_1 (43 kDa) and DSPA α_2 (39 kDa) show about 70–80% structural homology with human t-PA, but contain neither a kringle 2 domain nor a plasmin-sensitive cleavage site. DSPA β lacks the finger domain and DSPA γ lacks the finger and growth factor domains (23). DSPA α_1 and DSPA α_2 exhibit a specific activity *in vitro* that is equal to or higher than that of human rt-PA, a relative PAI-1 resistance and a greatly enhanced fibrin specificity with a strict requirement for polymeric fibrin as a cofactor (23). In several animal models of thrombolysis, DSPA α_1 (desmoteplase) has a 2.5 times higher potency and 4- to 8-fold slower clearance than rt-PA (24, 25). In patients with acute ischemic stroke, an elimination half-life of more than 2 h was reported, which may have a positive impact on reocclusion rates. Desmoteplase is a heterologous protein and may be antigenic in man.

Thus, several rt-PA variants have a significantly slower plasma clearance, allowing bolus administration. Their therapeutic potential is presently explored in patients with AMI, but also with ischemic stroke, pulmonary embolism, peripheral arterial occlusion or deep vein thrombosis. Furthermore, the addition of a variety of adjunctive agents to rt-PA and the use of extravascular angioplasty and stenting is explored (26–28).

Urokinase-type plasminogen activator

Urokinase (u-PA) is secreted as a 54 kDa single-chain molecule (scu-PA, pro-urokinase) that can be converted to a two-chain form (tcu-PA). u-PA is a serine proteinase of 411 amino acids, with active site triad His²⁰⁴, Asp²⁵⁵, and Ser³⁵⁶ located in the serine proteinase (COOH-terminal) domain. The molecule contains an NH₂-terminal growth factor domain and one kringle structure homologous to the kringles of plasminogen and t-PA (29); it contains only one N-glycosylation site (at Asn³⁰²), and is fucosylated at Thr¹⁸. Conversion of scu-PA to tcu-PA occurs after proteolytic cleavage at position Lys¹⁵⁸-Ile¹⁵⁹ by plasmin, but also by kallikrein, trypsin, cathepsin B, human T cell associated serine protease-1 and thermolysin. A fully active tcu-PA derivative is obtained after additional proteolysis by plasmin at position Lys¹³⁵-Lys¹³⁶. A low M_r form of scu-PA is generated by cleavage of the Glu¹⁴³-Leu¹⁴⁴ peptide bond. Recombinant scu-PA (sarpase) is expressed in *E. coli* and obtained as a 45 kDa non glycosylated molecule.

In contrast to tcu-PA, scu-PA displays very low activity toward low molecular weight chromogenic substrates, but it appears to have some intrinsic plasminogen activating potential, which represents $\leq 0.5\%$ of the catalytic efficiency of tcu-PA. In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen; however, in the presence of a fibrin clot, scu-PA, but not tcu-PA, induces fibrin-specific clot lysis (30, 31). This was explained by the finding that scu-PA is an inefficient activator of plasminogen bound to internal lysine residues on intact fibrin, but has a higher activity toward plasminogen bound to newly generated COOH-terminal lysine residues on partially degraded fibrin (32, 33).

Following intravenous infusion of recombinant scu-PA in AMI patients, a biphasic disappearance was observed with initial half-life in plasma of 8 min (34). Its therapeutic potential was investigated in several clinical studies in patients with AMI (including PRIMI [35, 36], SESAM [37], LIMITS [38], PATENT [39]) or with stroke (PROACT I and II [40, 41]).

Staphylokinase

Staphylokinase (Sak) is a 135 amino acid protein (comprising 45 charged amino acids, no cysteine residues nor glycosylation), secreted by *S. aureus* strains after lysogenic conversion or transformation with bacteriophages. The primary structure of Sak shows no homology with that of other plasminogen activators. Staphylokinase folds into a compact ellipsoid structure in which the core of the protein is composed exclusively of hydrophobic amino acids. It is folded into a mixed five-stranded, slightly twisted β -sheet which wraps around a central α -helix and has two additional short two-stranded β -sheets opposing the central sheet (42). Recombinant Sak is obtained by expression in *E. coli* (43, 44).

Sak forms a 1:1 stoichiometric complex with plasmin. It is not an enzyme, and generation of an active site in its equimolar

complex with plasminogen requires conversion of plasminogen to plasmin. In plasma, in the absence of fibrin, no significant amounts of plasmin are generated because traces of plasmin are inhibited by α_2 -antiplasmin. In the presence of fibrin, generation of the active complex is facilitated because traces of fibrin-bound plasmin are protected from α_2 -antiplasmin, and inhibition of the complex by α_2 -antiplasmin at the clot surface is delayed more than 100-fold. Furthermore, Sak does not bind to a significant extent to plasminogen in circulating plasma, but binds with high affinity to plasmin and to plasminogen which is bound to partially degraded fibrin (45–47).

Being a heterologous protein, Sak is immunogenic in man. Wild-type Sak contains three immunodominant epitopes. A comprehensive site-directed mutagenesis program resulted in the identification of variants with reduced antigenicity, but maintained fibrinolytic potency and fibrin-specificity, such as SakSTAR (K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K109A, K130T, K135R) (code SY 161) (48). Furthermore, SY 161 with Ser in position 3 mutated into Cys, was derivatized with maleimide-substituted polyethylene glycol (P) with molecular weights of 5,000 (P5), 10,000 (P10) or 20,000 (P20), and characterized *in vitro* and *in vivo* (49). Staphylokinase-related antigen following bolus injection of the P5, P10 or P20 derivatives in patients disappeared from plasma with an initial half-life of 13, 30 and 120 min and was cleared at a rate of 75, 43 and 8 ml/min, respectively, as compared to an initial half-life of 3 min and a clearance of 360 ml/min for wild-type staphylokinase (49).

Following several pilot trials in patients with AMI and peripheral arterial occlusion (46), the therapeutic potential of Sak and derivatives was studied in the CAPTORS I and II trials (50, 51).

Perspectives

Over the last two decades fibrin-specific thrombolytic agents have extensively and successfully been used for treatment of thromboembolic diseases. However, all currently used agents have significant shortcomings, including the need for large therapeutic doses, limited fibrin-specificity and significant associated bleeding tendency and reocclusion. Newly developed thrombolytic agents, mutants and variants of the serine proteinases tissue-type (t-PA) or urokinase-type (u-PA) plasminogen activator, have reduced plasma clearance and lower reactivity with proteinase inhibitors, and maintained or enhanced plasminogen activator potency and/or fibrin-specificity. The non-enzyme bacterial plasminogen activator staphylokinase has also shown promise for fibrin-specific thrombolysis, although neutralizing antibodies are elicited in most patients. The therapeutic potential of some of these agents is being evaluated in clinical trials. More widespread use of thrombolytic agents is presently limited by the more frequent application of mechanical interventions.

References

1. Tillett WS, Garner RL. Fibrinolytic activity of hemolytic streptococci. *J Exper Med* 1933; 58: 485–502.
2. Astrup T, Permin PM. Fibrinolysis in animal organism. *Nature* 1947; 159: 681–2.
3. Lack CH. Staphylokinase: an activator of plasma protease. *Nature* 1948; 161: 559–60.
4. Macfarlane RG, Pilling J. Fibrinolytic activity of normal urine. *Nature* 1947; 159: 779.
5. Williams JRB. The fibrinolytic activity of urine. *Br J Exper Pathol* 1951; 32: 530–7.
6. Bernik MB. Increased plasminogen activator (urokinase) in tissue culture after fibrin deposition. *J Clin Invest* 1973; 52: 823–34.
7. Nolan C, Hall L, Barlow G, et al. Plasminogen activator from human embryonic kidney cell cultures. Evidence for a proactivator. *Biochim Biophys Acta* 1977; 496: 384–400.
8. Collen D. On the regulation and control of fibrinolysis. *Thromb Haemost* 1980; 43: 77–89.
9. Collen D, Lijnen HR. Tissue-type plasminogen activator: a historical perspective and personal account. *J Thromb Haemost* 2004; 2: 541–6.
10. Pennica D, Holmes WE, Kohnr WJ, et al. Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature* 1983; 301: 214–21.
11. Lijnen HR, Collen D. Tissue-type plasminogen activator. In: *Handbook of proteolytic enzymes*, Barrett AJ, Rawlings ND, Woessner JF, eds, Academic Press, 1998; 184–90.
12. Hoylaerts M, Rijken DC, Lijnen HR, et al. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem* 1982; 257: 2912–9.
13. Thorsen S. The mechanism of plasminogen activation and the variability of the fibrin effector during tissue-type plasminogen activator-mediated fibrinolysis. *Ann NY Acad Sci* 1992; 667: 52–63.
14. The GUSTO Investigators. An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. *N Engl J Med* 1993; 329: 673–82.
15. GUSTO Angiographic Investigators. The effects of tissue plasminogen activator, streptokinase or both on coronary-artery patency, ventricular function, and survival after acute myocardial infarction. *N Engl J Med* 1993; 329: 1615–22.
16. Kohnert U, Rudolph R, Verheijen JH, et al. Biochemical properties of the kringle 2 and protease domains are maintained in the refolded t-PA deletion variant BM 06.022. *Prot Engineer* 1992; 5: 93–100.
17. The GUSTO-III investigators. A comparison of reteplase with alteplase for acute myocardial infarction. *N Engl J Med* 1997; 337: 1118–23.
18. Paoni NF, Keyt BA, Refino CJ, et al. A slow clearing, fibrin-specific, PAI-1 resistant variant of t-PA (T103N,KHRR296–299AAAA). *Thromb Haemost* 1993; 70: 307–12.
19. Guerra DR, Karha J, Gibson CM. Safety and efficacy of tenecteplase in acute myocardial infarction. *Expert Opin Pharmacother* 2003; 4: 791–8.
20. Den Heijer P, Vermeer F, Ambrosioni E, et al. Evaluation of a weight-adjusted single-bolus plasminogen activator in patients with myocardial infarction: a double-blind, randomized angiographic trial of lanoteplase versus alteplase. *Circulation* 1998; 98: 2117–25.
21. Suzuki S, Saito M, Suzuki N, et al. Thrombolytic properties of a novel modified human tissue-type plasminogen activator (E6010): a bolus injection of E6010 has equivalent potency of lysing young and aged canine coronary thrombi. *J Cardiovasc Pharmacol* 1991; 17: 738–46.
22. Katoh M, Suzuki Y, Miyamoto T. Biochemical and pharmacokinetic properties of YM866, a novel fibrinolytic agent. *Thromb Haemost* 1991; 65: 1193 (Abstract 1794).
23. Krätzschar J, Haendler B, Langer G. The plasminogen activator family from the salivary gland of the vampire bat *Desmodus rotundus*: cloning and expression. *Gene* 1991; 105: 229–37.
24. Lijnen HR, Collen D. Strategies for the improvement of thrombolytic agents. *Thromb Haemost* 1991; 66: 88–110.
25. Lijnen HR, Collen D. New thrombolytic strategies and agents. *Hematologica* 2000; 85 Suppl 2: 106–9.
26. Baker Jr WF. Thrombolytic therapy. *Clin Appl Thromb Hemost* 2002; 8: 291–314.
27. Keeley EC, Boura JA, Grines CL. Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials. *Lancet* 2003; 361: 13–20.
28. de Lemos JA, Gibson CM, Antman EM, et al. Abciximab and early adjunctive percutaneous coronary intervention are associated with improved ST-segment resolution after thrombolysis. Observations from the TIMI 14 trial. *Am Heart J* 2001; 141: 592–8.
29. Holmes WE, Pennica D, Blaber M, et al. Cloning and expression of the gene for pro-urokinase in *Escherichia coli*. *Biotechnology* 1985; 3: 923–9.
30. Gurewich V, Pannell R, Louie S, et al. Effective and fibrin-specific clot lysis by a zymogen precursor form of urokinase (pro-urokinase). A study in vitro and in two animal species. *J Clin Invest* 1984; 73: 1731–9.
31. Lijnen HR, Van Hoef B, Nelles L, et al. Plasminogen activation with single-chain urokinase-type plasminogen activator (scu-PA). Studies with active site mutagenized plasminogen (Ser740---->Ala) and plasmin-resistant scu-PA (Lys158---->Glu). *J Biol Chem* 1990; 265: 5232–6.
32. Liu JN, Gurewich V. Fragment E-2 from fibrin substantially enhances pro-urokinase-induced Glu-plasminogen activation. A kinetic study using the plasmin-resistant mutant pro-urokinase Ala-158-rpro-UK. *Biochemistry* 1992; 31: 6311–7.
33. Fleury V, Lijnen HR, Anglès-Cano E. Mechanism of the enhanced intrinsic activity of single-chain urokinase-type plasminogen activator during ongoing fibrinolysis. *J Biol Chem* 1993; 268: 18554–9.
34. Van de Werf F, Vanhaecke J, De Geest H, et al. Coronary thrombolysis with recombinant single-chain urokinase-type plasminogen activator (rs-cu-PA) in patients with acute myocardial infarction. *Circulation* 1986; 74: 1066–70.
35. PRIMI Trial Study Group. Randomised double-blind trial of recombinant pro-urokinase against streptokinase in acute myocardial infarction. *Lancet* 1989; 1: 863–8.
36. Spiecker M, Windeler J, Vermeer F, et al. Thrombolysis with saruplase versus streptokinase in acute myocardial infarction: five-years results of the PRIMI trial. *Am Heart J* 1999; 138: 518–24.
37. Bar FW, Meyer J, Vermeer F, et al. Comparison of saruplase and alteplase in acute myocardial infarction. SESAM Study Group. The Study in Europe with Saruplase and Alteplase in Myocardial Infarction. *Am J Cardiol* 1997; 79: 727–32.
38. Tebbe U, Windeler J, Boesl I, et al. On behalf of the LIMITS Study Group. Thrombolysis with recombinant unglycosylated single-chain urokinase-type plasminogen activator (Saruplase) in acute myocardial infarction: influence on early patency rate (LIMITS Study). *J Am Coll Cardiol* 1995; 26: 365–73.
39. Zarich SW, Kowalchuk GJ, Weaver WD, et al. Sequential combination thrombolytic therapy for acute myocardial infarction: results of the Pro-Urokinase and t-PA Enhancement of Thrombolysis (PATENT) trial. *J Am Coll Cardiol* 1995; 26: 374–9.
40. del Zoppo GJ, Higashida RT, Furlan AJ, et al. PROACT: a phase II randomized trial of recombinant pro-urokinase by direct arterial delivery in acute middle cerebral artery stroke. PROACT Investigators. *Stroke* 1998; 29: 4–11.
41. Furlan AJ, Abou-Chebi A. The role of recombinant pro-urokinase (r-pro-UK) and intra-arterial thrombolysis in acute ischaemic stroke: the PROACT trials. *Prolyse in Acute Cerebral Thromboembolism*. *Curr Med Res Opin* 2002; 18 Suppl 2: s44–7.
42. Rabijns A, De Bondt HL, De Ranter C. Three-dimensional structure of staphylokinase, a plasminogen activator with therapeutic potential. *Nat Struct Biol* 1997; 4: 357–60.
43. Behnke D, Gerlach D. Cloning and expression in *Escherichia coli*, *Bacillus subtilis* and *Streptococcus sanguis* of a gene for staphylokinase: a bacterial plasminogen activator. *Mol Gen Genet* 1987; 210: 52834.
44. Collen D, Zhao ZA, Holvoet P, et al. Primary structure and gene structure of staphylokinase. *Fibrinolysis* 1992; 6: 22631.
45. Lijnen HR, Collen D. Staphylokinase, a fibrin-specific bacterial plasminogen activator. *Fibrinolysis* 1996; 10: 119–26.
46. Collen D. Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. *Nat Med* 1998; 4: 279–84.
47. Collen D. The plasminogen (fibrinolytic) system. *Thromb Haemost* 1999; 82: 259–70.
48. Laroche Y, Heymans S, Capaert S, et al. Recombinant staphylokinase variants with reduced antigenicity due to elimination of B-lymphocyte epitopes. *Blood* 2000; 96: 1425–32.
49. Collen D, Sinnaeve P, Demarsin E, et al. Polyethylene glycol-derivatized cysteine-substitution variants of recombinant staphylokinase for single-bolus treatment of acute myocardial infarction. *Circulation* 2000; 102: 1766–72.
50. Armstrong P, Burton J, Palisaitis D, et al. Collaborative angiographic patency trial of recombinant staphylokinase (CAPTORS). *Am Heart J* 2000; 139: 820–3.
51. Armstrong PW, Burton J, Pakola S, et al. CAPTORS II Investigators. Collaborative Angiographic Patency Trial of Recombinant Staphylokinase (CAPTORS II). *Am Heart J* 2003; 146: 484–8.