Molecular Biology Reports
https://doi.org/10.1007/s11033-019-05167-x

REVIEW

Bacterial staphylokinase as a promising third-generation drug in the treatment for vascular occlusion

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Received: 21 May 2019 / Accepted: 29 October 2019

Abstract

Vascular occlusion is one of the major causes of mortality and morbidity. Blood vessel blockage can lead to thrombotic complications such as myocardial infarction, stroke, deep venous thrombosis, peripheral occlusive disease, and pulmonary embolism. Thrombolytic therapy currently aims to rectify this through the administration of recombinant tissue plasminogen activator. Research is underway to design an ideal thrombolytic drug with the lowest risk. Despite the potent clot lysis achievable using approved thrombolytic drugs such as alteplase, reteplase, streptokinase, tenecteplase, and some other fibrinolytic agents, there are some drawbacks, such as high production cost, systemic bleeding, intracranial hemorrhage, vessel re-occlusion by platelet-rich and retracted secondary clots, and non-fibrin specificity. In comparison, bacterial staphylokinase, is a new, small-size plasminogen activator, unlike bacterial streptokinase, it hinders the systemic degradation of fibrinogen and reduces the risk of severe hemorrhage. A fibrin-bound plasmin–staphylokinase complex shows high resistance to a_2-antiplasmin-related inhibition. Staphylokinase has the potential to be considered as a promising thrombolytic agent with properties of cost-effective production and the least side effects.

Keywords Clot · Myocardial infarction · Plasminogen activator · Staphylokinase · Thrombolytic therapy

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Published online: 01 November 2019

Introduction

Hemostasis is the arrest of bleeding from damaged vessels and is essential for survival. The main physiological processes involved, include: blood coagulation, platelet activation, and vascular contraction [1]. Blockage of blood flow is considered as one of the major causes of mortality [2]. In many countries, the majority of deaths and disabilities are caused by CVDs,[1] one of which comprises thrombotic disorders [3]. Every year, approximately 12 million people die after a MI or stroke [4]. Conditions associated with thrombosis, such as myocardial infarction, cerebral vascular thrombosis, pulmonary embolism, and DVT are a substantial threat to human life. Heart attacks and strokes often begin with blockage of the blood flow to the heart or brain due to atherosclerosis or the accumulation of blood components along the inner walls of the blood vessels, which reduces the velocity of blood flow in the vessels and decreases their flexibility [5]. Thereby, impeding the supply of blood to the brain and heart [5]. Platelet and thrombin play a major role in the development of thrombosis in the body and plasmin dissolve the resulting clot [6]. Fibrinolysis is a result of proteolytic degradation of fibrin by t-PA. Platelets induced by thrombin can make t-PA inactive through the production of PAI-1. As a result, a secondary clot is formed by platelet aggregation at the site of the initial clot lysis having thrombin [7]. Therefore, there is a need to design an antithrombotic agent that can inhibit the action of thrombin and platelet aggregation while causing the fibrinolysis of the blood clots.

Thrombolytic agents

Plasminogen protein is produced by liver as a pre-enzyme (zymogen) and is released in the blood. This protein is changed into a fibrinolytic enzyme called plasmin by plasminogen activators [8]. Hence, the intravenous infusion of plasminogen activators is an important strategy for treating thrombosis [6, 7]. As a result of plasminogen activation, several key bindings within the fibrin network are hydrolyzed, which dissolves clot [9]. Anticoagulants with blood-thinning effects like vitamin K antagonists (VKA) (the most widely used substance, warfarin,-a coumarin derivative—under brand name of Coumadin) and heparin as well as antiplatelet drugs such as aspirin have been widely used to treat and prevent clotting. [10, 11]. However, these products were only able to reduce the size of the clot while the rapid and complete dissolving of the clot would improve symptoms and save the patient's life [11, 12]. The history of therapeutic thrombotic drugs began in 1933, when the filtered fluid medium from a specific strain of *Streptococcus* (β-hemolytic *streptococci*) was discovered to dissolve the blood clot. The primary clinical application of streptokinase was in the fight against exudative pleural effusion, hemothorax, and tuberculous meningitis [13, 14]. In 1947, fibrinolytic potential of urine and its active molecule, called urokinase, identified for the first time. Unlike streptokinase, urokinase does not have antigenic properties [15]. In 1958, streptokinase (47.3 kDa) was first used in patients with AMI, which led to a focus on the treatment using this agent [16]. Unlike streptokinase, urokinase does not have such potent antigenic properties [15]. The t-PA is a natural fibrinolytic agent found in endothelial vascular cells [17]. It causes a balance between thrombolysis and thrombogenesis and has a high affinity for binding to fibrin [18]. Binding t-PA and plasminogen to the fibrin surface in the area of the thrombosis leads to a conformational changes, which facilitates the conversion of plasminogen to plasmin dissolving the clot [19]. Meanwhile, a third generation of thrombolytic drugs has reached the level of clinical trials. Many of them are derived from alteplase, which is currently the gold standard for the treatment of acute coronary syndrome. The most prominent are reteplase, tenecteplase, and lanoteplase (all from genetically mutated variants of alteplase) [3]. Treatment with thrombolytic agents, however, is not suitable for all patients. For example, delay in recovery in some patients following thrombolytic treatment after the onset of symptoms (rather than the early stages of disease) indicate that they are not eligible for this treatment [20]. Even, it may not be an appropriate treatment for those who were treated at early stages of disease. Further, increased risk of bleeding after treatment with thrombolytic drugs means that all patients need to be screened and evaluated for this type of treatment. However, the risk of bleeding in patients whose screening is good is not completely eliminated [20, 21]. Very severe bleeding can occur in the brain, and the consequent intracranial hemorrhage can be catastrophic. Therefore, the benefits and risks of thrombolytic treatment should be carefully considered [22].

The ideal thrombolytic drug should have the following characteristics: fibrin-specific, stable, enabling reperfusion, without reocclusion or formation of a secondary clot,

[1] The abbreviations used are: *CVDs* cardiovascular diseases, *DVT* deep venous thrombosis, *t-PA* tissue plasminogen activator, *PAI-1* plasminogen activator inhibitor type 1, *TIMI* thrombolysis in myocardial infarction, *ISIS* international study of infarct survival, *ASSENT* assessment of safety and efficacy of a new thrombolytic agent, *AMI* acute myocardial infarction, *AIS* acute ischemic stroke, *EGF* epidermal growth factor, *CHO* chinese hamster ovary, *DSPA* desmodus salivary plasminogen activators, *EACA* epsilon-aminocaproic acid, *LBS* lysine binding site, *FCB-2* fibrin(ogen) cyanogen bromide fragment-2, *E (DD)* complex of D-dimer non-covalently associated with fragment E, *DD* D-dimer, *SAK* staphylokinase, *PEG* poly ethylene glycol, *CAPTORS* collaborative angiographic patency trial of recombinant staphylokinase, *ESPRIT* european study of the prevention of reocclusion after initial thrombolysis.

resistance to PAI-1, no antigenicity, reasonable cost, easy administration, low incidence of systemic bleeding, and low incidence of intracranial hemorrhage [4, 23–25]. Non-fibrin-specific thrombolytic agents tend highly to plasma-soluble plasminogen converting it into plasmin while fibrin-specific thrombolytic agents have a lower tendency to this plasminogen [6]. The majority of plasminogen converted to plasmin is inhibited by α_2-antiplasmin. However, by decreasing α_2-antiplasmin, some of these plasmins, through the decomposition of fibrinogen, result in dissolving fibrinogen and an increased risk of intravenous hemorrhage. Specific thrombolytic agents for fibrin have a high tendency to make fibrin-bound plasminogen turn into plasmin. This plasmin is not available for α_2-antiplasmin and causes fibrin lysis. However, some of the plasmin remaining in the solution can also come into the clot area and cause fibrin degradation [6].

Plasminogen activators can either act directly or indirectly [22]. Direct activators of plasminogen act more specifically, with their protease-serine activity, they break down the Arg^{561}–$Va1^{562}$ band in the plasminogen and convert it into plasmin in the area of activity. Indirect activators such as staphylokinase (a promising third-generation drug) have no proteolytic activity and form a complex with a one–one stoichiometric ratio with plasminogen which can convert the next plasminogen into plasmin [22, 23].

First-generation thrombolytic drugs

First-generation drugs act systemically and are not selective for the clot; in fact, they are attached to both plasminogen in the blood and the clot.

Urokinase

Urokinase which is a two-chain urokinase-type plasminogen activator (tcu-PA), is a serine protease without fibrin specificity obtained from human urine or tissue culture of human kidneys [26].

This thrombolytic agent includes three domains: the serine protease domain at the end of the carboxyl, the kringle domain, and the growth factor domain at the end of amino [26]. Severe side effects have been reported after the administration of urokinase to patients with AMI, including the transmission of infectious agents during the production process. For this reason, the FDA has withdrawn abbokinase (commercially available urokinase) from the market [27].

Streptokinase

Streptokinase is a plasminogen activator produced by various strains of β-hemolytic *streptococci*. Streptokinase is a non-fibrin specific extracellular enzyme that performs its fibrinolytic activity by indirect activation of plasminogen available in blood circulation [28]. Like urokinase, streptokinase has three domains: alpha, beta, and gamma-which are separated by two linkers. The half-life of streptokinase is approximately 30 min [29, 30]. Administration of this prokaryotic protein is accompanied by allergic reactions andhypotension, in addition, there is also an antibody-dependent inhibition of streptokinase (preventing its reuse) [23, 31]. Due to the systemic plasminogen activation, the administration of this protein also causes bleeding problems (extensive fibrinogen depletion) and formation of a secondary clot [28].

Approximately 30% of patients suffering from AMI who treated with streptokinase was classified as TIMI grade 3 flow (complete perfusion) by 90 min and 20% of them achieved TIMI flow grade 2 flow (partial perfusion). A 30 day mortality rate of 7.3% and an intracranial hemorrhage rate of 0.54% was observed [32].

Second generation thrombolytic drugs

Second-generation drugs are selective for the clot and tend more to plasminogen attached to clots.

Saruplase

Saruplase is a recombinant single-chain urokinase-plasminogen activator (r-scu-PA) or prourokinase. This protein is actually a naturally occurring prodrug from a protease. Under in vivo condition, this protein relatively changes to its active form by plasmin—i.e. low-molecular-weight double-stranded urokinase (276 amino acids) [33]. In addition, the unconverted saruplase component can directly activate plasminogen [32]. The half-life of saruplase is low (7–8 min) [33]. Saruplase reduces systemic plasmin in patients. In addition, its consumption is accompanied by a decrease in α_2-antiplasmin and fibrinogen and an increase in fibrinogen-degradation products [34]. Its systemic fibrinolytic activity is less than that of streptokinase but more than that of alteplase. The fibrin-specific binding in saruplase is lower than that of alteplase [35].

Recombinant tissue plasminogen activator

Human t-PA was originally obtained from the human uterus [36]. It is a glycoprotein with four domains located N-terminally (finger (F-domain) (residues 4–50), epidermal growth factor (E-domain) (residues 50–87), kringle1 (K1 domain) (residues 87–176), kringle2 (K2 domain) (residues 176–256)) and a domain C-terminally (catalytic serine protease (P- domain) (residues 276–527)), 17 di-sulfide bonds, and a molecular weight of 67 kDa—is important as it creates a balance between the phenomenon of thrombolysis and thrombogenes in the fibrinolytic system [36–38].

Catalytic triad contain His^{322}, Asp^{371}, and Ser^{478}. Both F and K2 domains bind to fibrin and accelerate the activation of plasminogen by t-PA [36]. The plasma half-life of native t-PA is short due to liver clearance [39]. This molecule has three potential sites of N-glycosylation at residues of 117, 184 and 448. It also includes an O-linked fucose residue at Thr^{61}. The fast clearance of t-PA in the hepatocytes is due to E and F domains as well as carbohydrate side chain residues [40]. There may be two phases of t-PA clearance rate from the hepatocytes: (1) rapid clearance by E and F domains interaction (2) the second phase caused by glycosylation sites presence [41]. Mannose receptors in the liver recognize Asn^{117} residue in the K2 domain that causes its rapid clearance. Moreover, t-PA without the E domain indicated an enhanced in vivo half-life [40]. Its reduced clearance rate is due to replacement of Tyr^{67} in the E-domain. Amino acid changes in the F domain between residues 42–49 also have an important role in drug clearance [42]. t-PA is the main plasminogen activator in the blood while u-PA carries proteolytic activity in the tissue and has a lower effect on the intravascular lysis of fibrin as compared to t-PA [43]. For beginning and extending the fibrinolysis process the sedimentation of a large amount of plasminogen and t-PA in fibrin deposits occur, along with increase of plasmin activity in this section [6]. T-PAs are available in two forms—single chain forms of sct-PA and two-chain tct-PA. PAI-1 inhibits the plasminogen activation activity of t-PA [38]. PAI-1 is the most effective inhibitor of t-PA and is a serine protease inhibitor (serpin) that acts as a false substrate for its target protease, with which it forms an inactive equimolar complex. This t-PA inhibitor acts through forming a complex between the active site of t-PA and the Arg^{346}–Met^{347}, trapping amino acids in its structure. The PAI-I is in two forms—active and inactive. The active form loses its activity spontaneously and has a half-life of about half an hour. The inactive form is the result of active form decomposition. PAI-I is made by endothelial cells and hepatocytes and it is seen in platelets, placenta, and serum [4].

Alteplase (rt-PA) is a tissue plasminogen activator manufactured using rDNA technology and using the cDNA of human melanoma cell lines [44]. Clinical trials have confirmed the efficacy and safety of rt-PA in the treatment of occlusions led by vessel-related devices such as catheters [45]. Intravenous alteplase is superior to placebo for both AMI and AIS if used early [46]. Thrombolysis with alteplase < 12 h after AMI had a great impact and its maximum benefit was within < 6 h [46]. Effect of intravenous alteplase plus heparin within 5 h from onset of symptoms into patients with suspected AMI showed the 6 months mortality rate of 10.4% for alteplase compared with 13.1% of that placebo, a relative reduction of 21%. Also, patients with proven MI revealed a relative reduction of 26% [47]. Comparatively, alteplase < 3 h of the onset of stroke significantly enhanced the chance of an almost complete recovery in the people [48]. Among patients treated between 3 and 4.5 h after the onset of a stroke, the rate of adverse outcome (death or severe disability) in alteplase group was significantly lower than that of placebo (48 vs. 55%; $P = 0.04$) [46]. Alteplase infusion within 6 h of ischemic stroke exhibited a significantly higher hazard of death during the first 7 days. However, those who survived the acute phase, showed a significantly long-term survival [49]. In a concentration-dependent manner, the incidence of symptomatic intracranial hemorrhage was higher with alteplase than with placebo (2.4 vs. 0.2%; $P = 0.008$) [49, 50].

Anistreplase

Anistreplase, is also known as anisoylated plasminogen-streptokinase activator complex, is actually another form of streptokinase [9, 40]. This complex consists of human plasminogen and acylated streptokinase conjugated with an equal molecular concentration, which—after spontaneously deacylation—converts the next plasminogen into plasmin. The anistreplase deacylation (hydrolysis) leads to the release of the p-anisoyl group [51]. This activator complex is a fibrin non-specific binding thrombolytic agent with side effect profile similar to that of streptokinase, but has the advantage of single-bolus administration [52]. Administration of anistreplase led to a combined TIMI grade 2 and TIMI grade 3 flow rate of 50–60% at 90 min. The ISIS–3 study reported a mortality rate of 10.5% at 35 days and an intracranial hemorrhage of 0.6% after once anistreplase injection [31, 53].

Third-generation thrombolytic drugs

Third-generation drugs show high propensity to clot-bound plasminogen instead of plasma plasminogen even more so than for the second-generation drugs [54, 55].

Tenecteplase

Tenecteplase (TNK-tPA) is a mutated rt-PA in three sites in which the half-life of protein is increased up to 5–6 times; it represents bindings 80 times lower than that of alteplase to PAI-1 [56]. Tenecteplase, similar to the alteplase, is bound to the fibrin [57]. However, this specificity is 15 times more than that of alteplase [56]. Its usable dosage is 0.25 mg/kg as a single bolus [56]. The ASSENT-1 clinical trial demonstrates the efficacy and safety of tenecteplase [58]. However, the ASSENT-2 clinical trial in patients with AMI reported that hemorrhage and allergic reactions were associated with this drug [59].

Reteplase

Reteplase is a single-chain mutant t-PA in which three EGF, finger, and kringle-1 domains have been removed [35]. It is a non-glycosylated protein compared to alteplase, expressed in *Escherichia coli* bacteria [60]. Reteplase, like alteplase, demonstrates increased plasminogen activation in the presence of fibrin. However, the tendency of binding to fibrin is significantly (5 times) less than that of alteplase [61]. This thrombolytic agent has a high half-life (18 min) (approximately 4 times that of alteplase). Its usable dose is 10 milliunits, plus 10 milliunits, as oral administration every half hour [35]. Reteplase eligibility has been evaluated in a clinical trial in patients with AMI. It was shown that dual administration of reteplase versus the standard dose of alteplase can cause earlier and more vasodilatation [62]. Similar to other drugs, hemorrhage and allergic reactions are associated with the use of reteplase [63].

Monteplase

Monteplase is a fibrin-specific binding tissue plasminogen activator. This mutated protein is an improved thrombolytic agent for a clinical application in which amino acid cysteine at position 84 of the growth factor domain is substituted by serine [60, 61]. The biological half-life of monteplase is greater than that of the alteplase. This thrombolytic agent can be injected as a single bolus injection (in about 2 minutes) while another mutated t-PA requires intra-arterial injection for more than 60 min [61].

Lanoteplase

Lanoteplase (nPA) is a wild-type mutated t-PA in which the domains for EGF and t-PA finger have been removed and the kringle 1-glycosylated points have also been changed. This thrombolytic agent, produced in CHO cells, has a half-life of approximately 10 times of that of alteplase and can reach up to 45 min [24, 64]. In animal models, it has more lytic activity and less fibrin tendency than alteplase [35]. Kidney clearance abilities and the optimal half-life of this molecule make it suitable for single administration. Its usable dose is 120 kilounit per kilogram [24, 35]. Because of an increased incidence of intracranial hemorrhage, it was not licensed in the U.S [65, 66].

Pamiteplase

Pamiteplase or sulinase is another mutated t-PA. Its kringle 1-glycosylated domain are removed and it has a single-point mutation (Arg^{275} to Glu) leading it resistant to conversion to a two-chain form by plasmin. Pamiteplase and t-PA have the same fibrin tendency and the same specific activity in the in vitro conditions. In addition, inhibition of pamiteplase and t-PA by PAI-1 is similar [67]. Plasma clearance of pamiteplase was estimated to be 7 times slower than t-PA in pharmacokinetic properties on rats. Mean residence time or lifetime (Half-life = 0.693 × (Residence time) [68] for pamiteplase was 62 min versus 9 min for t-PA [69]. Pamiteplase showed more thrombolytic effect after intravenous injection than t-PA in a canine model of coronary artery thrombosis [69]. After 30 min of thrombosis induction in rabbit, comparison of single bolus intravenous administration of pamiteplase and single bolus or 60 min infusion of t-PA showed that thrombolytic activity of pamiteplase was 4 times more than that of t-PA, though dose-dependent thrombolytic activity was observed in both of them. The better thrombolytic activity of pamiteplase was associated with its relatively higher value in plasma due to its longer half-life. The clearance of plasma fibrinogen to less than 20% of the baseline level was observed in all groups [70]. The bleeding time also did not change significantly in any group [70, 71]. In addition to plasma fibrinogen depletion [70], toxicity studies of pamiteplase on rats and monkeys totally showed a hemorrhage, increase of coagulation time and transient decrease in locomotor activity at the injection sites. Other side effects were increased platelet count, partial reduction of hemoglobin and hematocrit, increased level of plasma phospholipids, total protein, total cholesterol, and liver weight. The approximate single lethal dose of pamiteplase was more than 60 mg/kg in rats, and more than 10 mg/kg in squirrel monkeys and cynomolgus monkeys [72]. In a pharmacokinetic study in human volunteers, biological activity (lysis time of 50% of the clot) was 30–47 min after a single bolus administration of 0.5–4 mg/kg pamiteplase [67]. In another study, in 157 patients with AMI treated with 0.05, 0.1, 0.2, or 0.3 mg/kg of pamiteplase, complete patency of the vessel (TIMI grade 3 flow rates) after 60 min was observed in 42%, 57%, 63%, and 54% of the patients respectively. Adverse effects were also observed in 7 and 17% of patients receiving 0.2 and 0.3 mg/kg of the drug respectively [69].

Duteplase

Duteplase (met-t-PA) is a third-generation 2-chain t-PA in which valine at position 245 is substituted by methionine [42, 73]. It is produced in CHO cells. In vitro specific activity of duteplase is lower as compared to alteplase [40]. Duteplase possesses slower clearance rate than single chain t-PA [74]. The proteolytic cleavage should make duteplase susceptible to irreversible inactivation. Because of reduced thermal stability, met−t-PA exhibits a lower rate of serious and fatal bleeding side effects [42]. It was found that a weight adjusted injection of duteplase combined with oral aspirin and intravenous heparin for AMI could be as

effective and safe as alteplase in the ESPRIT study. But following the initial successful thrombolysis, reocclusion and reinfarction was a problem [75].

Amediplase

Amediplase (K2tu-PA) consists of the first 3 residues of the F domain and the K2 domain of t-PA (1–3 + 176–275) linked to the catalytic P domain of scu-PA (159–411) [42]. The molar mass of the third-generation thrombolytic agent produced in CHO cells is about 39.9 kDa (356-amino-acid) [40]. Plasma half-life was 30 min after bolus injection in rabbits. The reason is probably the lack of F, EGF and K1 domains [74]. Amediplase showed a tenfold lower activity than alteplase in a clot-internalized lysis model (in-clot injection) but this activity was similar in an external model [76]. This result suggested that amdiplase had weak fibrin affinity due to the interaction of the P domain from the urokinase with the K t-PA domain. However, it indicated a better clot penetration [39, 40, 74]. In another model of external clot lysis, amediplase at therapeutic concentrations was more active than TNK-tPA and scu-PA [77].

Desmoteplase

The saliva of the vampire bat *Desmo dusrotundus* contains a family of four plasminogen activators known as DSPA, among which DSPA alpha 1 or Desmoteplase (bat-PA) is larger than the others (477 amino acids) and is structurally similar to t-PA in human. It includes a finger domain, an EGF domain, and a single kringle domain, without having a secondary kringle or a plasmin cleavage site, which is essential for conversion into a two-chain form of t-PA [78, 79]. Not converting to a two-chain form makes bat-PA the only native single-chain plasminogen activator with complete catalytic activity [79]. The bat-PA is produced by using recombinant technology in mammalian cells. This protein, as a thrombolytic agent, is highly fibrin-specific and performs its fibrinolytic activity by the direct activation of plasminogen [9, 40]. Its usable dose is 0.5 mg/kg [35]. While bleeding time (BT) was similar to that of human t-PA, bat-PA caused a much higher number of long-term bleeding events than t-PA [80]. In the only clinical trial of bat-PA reported so far, the half-life of the drug was 2.8 h, which was suitable for single bolus administration in clinical use [35]. However, the bat-PA stimulated the immune system [35].

Other plasminogen activators

Plasminogen activators from snakes

Venom of snake composed of very different types of proteases with fibrinolytic properties. *Trimeresurus stejnegeri* venom plasminogen activator, TSV-PA (234 amino acids, approximate molar mass of 33 kDa and pI of 5.2) is a single-chain plasminogen activator obtained from the venom of the chinese green tree viper snake [81]. Moreover, venom of this snake contains enzymes like stejnobin and stejnefibrase 1–3. These enzymes directly degrade fibrinogen without plasminogen activator activity. The TSV-PA includes a single potential site of N-glycosylation at Asn^{161} and 6 disulfide bonds in addition to His^{41}, Asp^{86} and Ser^{180} considered as the catalytic triad [40, 81]. The TSV-PA glycoprotein has only 21–23% sequence similarity with the catalytic domains of u-PA and t-PA without presence of sequences responsible for interaction of t-PA (KHRR; Lys-His-Arg–Arg) and u-PA (RRHR; Arg–Arg-His-Arg) with PAI-1. As a results, it lead to the prolonged half-life [40, 42]. *E. coli* has been used to produce TSV-PA [81].Considering the lack of the presence of F, epidermal growth factor and K domains, it can be said that the plasminogen activator activity is not increased in the presence of fibrin (nearly 50-fold < that of t-PA) [42].

Haly-PA, a glycoprotein with 234 amino acids (32 kDa) purified from the venom of *Agkistrodon halys*, is 82% similar to the TSV-PA [82]. Expression system of baculovirus has been used to express this molecule [40]. The activity of this plasminogen activator is 30-fold lower than that of u-PA. The prolonged half-life of Haly-PA is because of the lack of domains for interaction with PAI-1 [42].

Moreover, another plasminogen activator, i.e.LV-PA (33 kDa) were purified from a snake (*Lachesis muta*) with a 90% sequence identity with TSV-PA and 85% identity with Haly-PA [83].

Chimeric plasminogen activators

A specific activity of 150,000 IU/mg was observed with a combination of GHRP (Gly-His-Arg-Pro) tetrapeptide and 32 kDa low-molecular-weight scu-PA (144–411). Other properties of molecule (GHRP-scu-PA-32 K) expressed in CHO cells were a 2.5-fold higher fibrin binding, higher thrombolytic potency and lower fibrinogen depletion in plasma as compared with the native low molecular weight scu-PA [84].

A new chimeric truncated t-PA variant (394 amino acids) was also created that called GHRP-SYQ-K2S. It included the kringle 2 domain and the serine protease domain (K2S) [85]. The domains of F, epidermal growth factor and K1 were deleted just like reteplase. The first 3 residues of t-PA (Ser-Tyr-Gln) were kept due to their importance for protein activity. A chimeric tetrapeptide (Gly-His-Arg-Pro) was also added upstream of SYQ-K2S for compensation of the loss of fibrin affinity as a result of the deletion of the finger domain. With a yield of 752 IU/mL (566,917 IU/mg), it was produced in CHO cells [42, 85]. There was a possibility of GHRP-SYQ-K2S interaction with fibrin monomers and

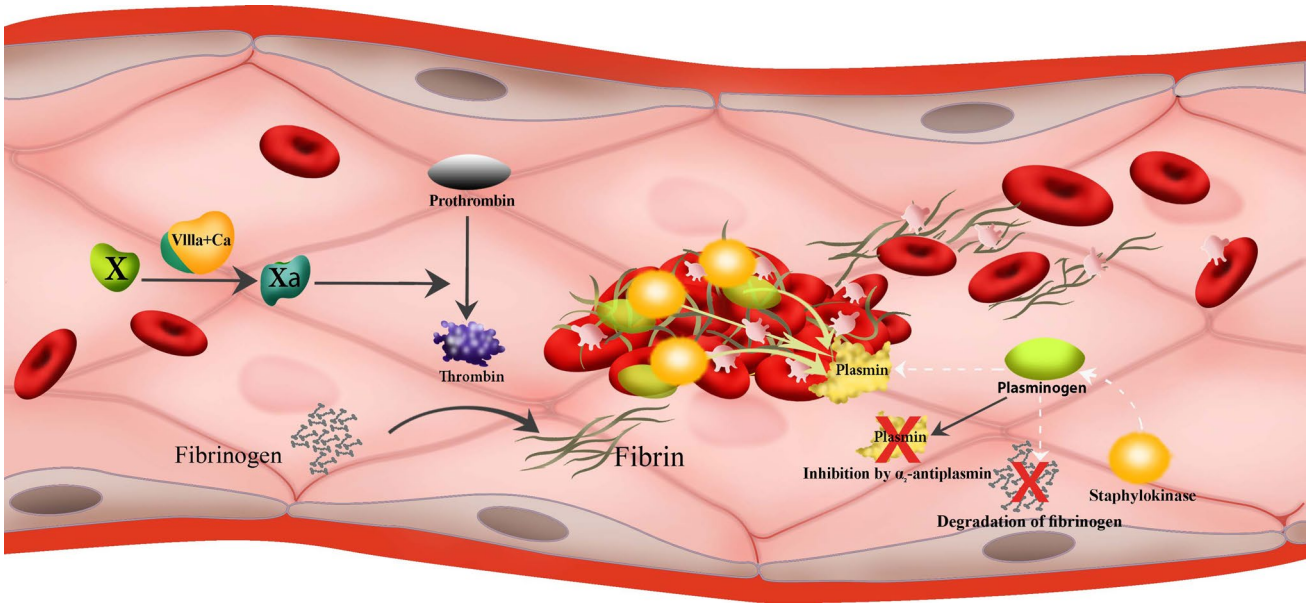

Fig. 1 Mechanism of the action of staphylokinase for dissolving the clot. Fibrin-specific thrombolytic agents such as staphylokinase have a lower tendency to plasma-soluble plasminogen converting it into plasmin. The majority of soluble plasmin is inhibited by α_2-antiplasmin. By decreasing α_2-antiplasmin, some of these plasmins, result in dissolving fibrinogen and an increased risk of intravenous hemorrhage. Staphylokinase is effective in terms of dissolving the clot. It have a high tendency to make fibrin-bound plasminogen turn into plasmin. This plasmin is not available for α_2-antiplasmin and causes fibrin lysis. However, some of the plasmin remaining in the solution can also come into the clot and cause fibrin degradation. Arrows with dash indicates the fewer efficacies of the action

polymerization hindrance. According to In vitro studies, the molecule has 86% of the fibrin binding capacity of t-PA in comparison with 30% for reteplase [40, 85].

Combination of plasminogen activator with anti-aggregation of platelet (a decorsin as platelet aggregation inhibitor, a low molecular weight scuPA-33 kDa and a thrombin inhibitory domain) [86] or its combination with antibodies against fibrin [86] was also provided.

Staphylokinase

Thrombolytic agents mentioned above have some side effects, including hemorrhage, vessel re-blockage, or high immunogenicity. The use of bacterial staphylokinase with small size (15.5 kDa) in the treatment and removal of blood clots was considered for a wide range of vascular system disorders. Indeed, it is a promising third-generation thrombolytic agent with fibrin specificity that can be used potentially for these disorders [4] (Fig. 1).

History of staphylokinase purification

Initially, Davidson (1951) and Glanville (1963) precipitated protein containing staphylokinase from supernatant fluid of cultures by adjusting the pH to 3.3 with ~ 10 mM HCl [87, 88]. Glanville also precipitated staphylokinase at 75% saturation of $(NH_4)_2S0_4$. The in vitro fibrinolytic properties of staphylokinase were evaluated by Lewis (1964) and Sweet (1965) [89, 90]. Lewis (1964) and Kanae (1986) studied the in vivo thrombolytic of staphylokinase in dogs [91, 92]. Jackson and Kondo (1981) purified the staphylokinase by affinity chromatography on plasmin-Sepharose [93] and plasminogen-Sepharose [94].

Characteristics of staphylokinase

The half-life in plasma of staphylokinase is 6 min [35]. It is a protein found in the culture medium of many strains of *Staphylococcus aureus* which could convert passive plasminogen into active plasmin [95, 96]. Plasminogen activation of staphylokinase is carried out by a two-step mechanism [97]. First, a complex between staphylokinase and plasminogen occurs, then the active region of this complex is accessible to convert plasminogen into plasmin. In fact, by forming this complex, peptide bond between lysine 10 and lysine 11 in staphylokinase is hydrolyzed, which ultimately results in a peptide bond cleavage between arginine 561 and valine 562 of plasminogen [6]. In the initial delay phase, plasminogen in the staphylokinase-plasminogen complex converts to plasmin [98]. After formation of a small amount of plasmin, staphylokinase binds to plasmin instead of plasminogen, and then the staphylokinase-plasmin complex rapidly and directly transforms plasminogen into plasmin. The activation effect of the staphylokinase-plasmin or plasminogen

complex is inhibited by α_2-antiplasmin in the absence of fibrin [99, 100]. The fibrin-bonded staphylokinase-plasmin complex is about 100 times more resistant to α_2-antiplasmin than the complex without fibrin bond [6]. The inhibition of the staphylokinase-plasmin or plasminogen complex via α_2-antiplasmin is suppressed by EACA, which is in fact similar to LBS of plasmin [101]. This inhibitory effect is reduced in the presence of fibrin or FCB-2 (composed of chain remnants Aα148-207, Bβ191-224, 225-242, 243-305, γ795-265, linked by disulfide bond) by competition for binding to LBS. The initial rate of activation of plasminogen by staphylokinase increases 2 to 3 times in the presence of fibrin [102]. Staphylokinase alone does not bind to the fibrin, but the staphylokinase–plasminogen or plasmin complex is bound to fibrin by LBS [4]. E (DD) is a complex of d-dimer non-covalently associated with fragment E. This terminal plasmin digestion product of fully cross-linked fibrin binds t-PA and plasminogen with affinities to the same extent as fibrin. It is shown by using the complex E (DD) the enzymatic activity (plasminogen activation) of staphylokinase is increased up to 38 times [103].

Although, staphylokinase is highly fibrin-specific (Table 1), but it has fewer fibrinogenolytic properties than streptokinase. Systemic plasminogen degradation, α_2-antiplasmin consumption, and systemic fibrinogen activation using staphylokinase have not been observed [4]. The dosage is 15 mg for double bolus administration every half hour. Its production can be made easier due to its small size [35]. The optimized form of SAK sequence variant, which was called THR-174 and produced by ThromboGenics NV [104], indicated increased efficacy and safety profiles in the pre-clinical trials. Moreover, a significant decrease observed in immunogenicity in comparison with streptokinase and other staphylokinase variants.

Staphylokinase protein structure

X-ray refraction, dynamic light refraction, ultra-centrifuge, and UV circular dichroism spectroscopy indicate the solution structure of staphylokinase. Rotational radius, radiation radius, maximum size, and sedimentation coefficient are 2.3 nm, 2.12 nm, 10 nm, and 1.71 S, respectively, which represent an elongated form of staphylokinase [4]. This single-chain polypeptide has a molecular weight of 15.5 kD [105] and does not have any significant homology with streptokinase [106]. Staphylokinase comprises two folded domains with the same size. The mean value of the distance between domain's centers of gravity equals 3.7 nm. In this solution, the mutual position in two domains is variable. That is why the shape of this molecule is like a flexible dumbbell [107]. According to electrophoresis on SDS-PAGE and isoelectric point, several molecular forms of different molecular weight of staphylokinase have been identified [108, 109]. Apparently, low molecular weight forms of mature staphylokinase lack amino-terminal residues. In a buffer environment, the interaction of plasminogen or plasmin with mature staphylokinase converts this protein into Sak-ΔN10 with the first ten residues truncated [110]. It has been shown that Sak-ΔN10 has a fibrinolytic activity similar to staphylokinase. It was also found that amino acid at position 26 is useful for the activation of plasminogen by staphylokinase. Replacing this amino acid with arginine or valine leads to a lack of functional activity but replacing it with leucine or cysteine has little or no effect on the functional activity [110].

Knowledge of the three-dimensional structure of a protein permits the design of PEG attachment site(s) via computer modeling. Amino acid substitutions such as K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K109A, K130T, and K135R were carried out along with substitution the Ser, found naturally at position 3 of staphylokinase, mutated into Cys. Then, the Cys was mono-PEGylated with different molecules of maleimide–PEG in molecular weights of 5, 10 or 20 kDa (namely SY 161-P5 or P10 or P20) which reduced antigenicity and prolonged plasma half-life [4, 111–115]. Recently, the N-terminal lipid modification of staphylokinase has enhanced the stability and activity of the SAK. This modification can help to its translocation across blood brain barriers and treatment of diseases like stroke [116].

Staphylokinase with thrombolytic, anti-platelet and anti-thrombin activities

Recent studies prove that activated platelets play a key role in thrombosis, formation of secondary clots and vessels re-blocking. After thrombolytic treatment, the secondary clot is formed due to platelet aggregation [117]. When lysis of the clot occurs, the coagulation system becomes active, in addition activation and aggregation of platelets take place due to the release of large amounts of thrombin. Subsequently, the activated platelets inhibit fibrin lysis by tissue plasminogen activator via releasing the type 1 plasminogen activator inhibitor (PAI-1) in the blood circulation. Therefore, it leads to the re-blockage of the vessels [7]. Arginine, lysine and aspartic acid tripeptide (RGD) attached to the staphylokinase can bind to the glycoprotein membrane receptor (GPIIb/IIIa) at the platelet surface and prevent fibrinogen binding to this receptor which results in no accumulation of activated platelets [6]. Snake venom [118] and F(ab')2 fragment of the monoclonal antibody 7E3 also pose as platelet inhibitors [119].

Various thrombin inhibitors such as hirudin and its derivatives can be added to the recombinant staphylokinase to decrease the formation of secondary clot [120, 121]. Hirudin, obtained from leech saliva, is a potential inhibitor of the

Table 1 Some key characteristics of the different generations of plasminogen activators

Generation	Name	Origin	Fibrin specificity	Immunogenicity	Plasma half-life (min)	Plasminogen Activation	Approved for clinical use
First- generation thrombolytic agents	Urokinase	Human urine and kidney tissue culture	No	No	15	Direct	Yes
	Streptokinase	β hemolytic streptoccus	No	Yes	30	Indirect	Yes
Second-generation thrombolytic agents	Alteplase	Vascular endothelial cells	++	No	4–6	Direct	Yes
	Saruplase	Renal cells	+	No	7–8	Direct	–
	Anistreplase	Anisoylated plasminogen-streptokinase activator complex	No	Yes	90–112	Direct	Yes
Third-generation thrombolytic agents	Tenecteplase	t-PA mutant	+++	No	20	Direct	Yes
	Reteplase	t-PA mutant	+	No	18	Direct	Yes
	Monteplase	t-PA mutant	+	[b]	23	Direct	–
	Lanoteplase	t-PA mutant	+	No	45	Direct	–
	Pamiteplase	t-PA mutant	++	[b]	30-47	Direct	–
	Duteplase	t-PA mutant	Yes[a]	[b]	05-Oct	Direct	–
	Amediplase	t-PA mutant	Yes[a]	[b]	30	Direct	–
	Desmoteplase	Vampire bat salivary	+++	Yes	128	Direct	–
	Staphylokinase	Staphylococcus aureus	+++	Yes	6	Indirect	–
Other plasminogen activators	TSV-PA	Trimeresurus Stejnegeri	Resistance to plasminogen activator inhibitor-1 (PAI-1) lead to prolonged plasma half-life. Plasminogen activator activity is not increased in the presence of fibrin (Nearly 50-fold < that of t-PA)			Direct	–
	Haly-PA	Agkistrodon halys	Resistance to PAI-1 lead to prolonged plasma half-life. It has 30-fold lower plasminogen activator activity than that of u-PA			Direct	–
	LV-PA	Lachesis muta	There is 90% sequence identity with TSV-PA and 85% with Haly-PA			Direct	–
	GHRP-scu-PA-32 K	Chimeric low- molecular- weight scu-PA	As compared to the native low molecular weight scu-PA, it shows a higher fibrin binding (2.5-fold), higher thrombolytic potency and lower fibrinogen depletion			Direct	–
	GHRP-SYQ-K2S	Chimeric truncated t-PA	It interacts with fibrin monomers and hinders polymerization. The molecule has 86% of the fibrin binding capacity of t-PA			Direct	–

[a]Data about the level of fibrin specificity is not available

[b]Data is not available

thrombin-specific serine proteases which forms a stable non-covalent complex with α-thrombin and prevents fibrinogen cleavage and fibrin formation by thrombin [120]. Hirudin consists of 63 amino acids with 3 sulfide bonds plus one O-glycosylated position in amino acid 45 and one modified amino acid in the position 62 called sulfotyrosin. This inhibitor also plays a role in preventing the accumulation of platelet associated with thrombin [120]. Components derived from hirudin such as 20 amino acids sequence (hirulog) [6], amino acid 12 fragment of hirudin (HV) [122], hirogen (N-acetyl hirudin 53′-64′ with sulfato-Tyr^{63}) and hirulog 1 (d-Phe-Pro-Arg-Pro-(Gly)4 desulfato-Tyr63′-hirugen) [123] also play an important role in thrombin inhibition. Other thrombin inhibitors include fibrinopeptide A [124], dipetalin domains [125] and tsetse thrombin inhibitors (TTI) [126, 127]. In addition, there are thrombin production inhibitors such as the recombinant anti-coagulant peptide of ticks and bed bugs [128] and recombinant activated protein [129].

Expression of staphylokinase in different hosts

The SAK gene has been cloned and expressed to varied levels in different expression systems like IPTG inducible *E.coli* BL21 [130, 131], salt induced *E.coli* GJ1158 [6], salt induced *E. coli* strain (DH5α) [132], IPTG containing *E.coli* JM 109 (DE3) [133], *Bacillus subtilis* [134, 135], methanol inducible yeast of *pichia pastoris* strain GS115 [7, 30, 136, 137], *Streptomyces lividans* [138], methylotrophic yeast *Hansenula polymorpha* [139] and under control of various *E.coli* promoters viz., *T7*, lambda *PR*, *tac* and *ptac*, *B. subtilis* promoters of *P43*, *Pamy* and *PsacB*, and *P. pastoris* AOX1 promoter.

Yeasts of *S. cerevisiae* and *P. pastoris* have a majority of N-linked glycosylation of the high-mannose type [140]. Prevention of hyper-glycosylation in the *H. polymorpha* and *P. pastoris* becomes an additional advantage of these expression systems over the traditional *S. cerevisiae* system [140, 141]. Moreover, *S. cerevisiae* core oligosaccharides have terminal α-1,3 glycan linkages leading the hyper-antigenic nature of these proteins while *P. pastoris* may be similar to the glycoprotein structure of higher eukaryotes [142].

Drawback of N-glycosylation of exotic proteins with therapeutic usage like SAK originated from a prokaryote (*S. aureus*), expressed in *P. pastoris* could be resolved by using purification by concanavalin A column chromatography [143] or treatment with Endo H [139] or adding glycosylation inhibitors such as tunicamycin [137].

The levels of expression and characteristics of staphylokinase or its multi-functional derivatives have been compared in various expression systems (Table 2). In this regard, the staphylokinase gene isolated from lysogenic *S. aureus* were cloned in pET-28a or pRSET-A and expressed in IPTG inducible *E. coli strain BL21* [144–148] and salt inducible *E. coli* GJ1158 [6]. The recombinant and mature staphylokinase protein expressed in *E. coli* was extracted and analyzed by various methods and its thrombolytic activities were confirmed. In *E. coli* BL21, the expression of recombinant staphylokinase led to the formation of inclusion bodies. Lee et al. (1998) obtained 20 mg/L of staphylokinase from the periplasmic space of *E. coli* [147]. Schlott et al. (1994) obtained 200 mg of staphylokinase per liter of fermentation broth [149]. In another study, Nguyen et al. (2014) obtained 300 mg of staphylokinase per liter of culture medium [148]. Other expression levels included 70–500 mg/L suggested by Schlott et al. (1999) [150] and 2.8 g/L of fermentation broth, as suggested by Mandi et al. (2009) [151]. Ye et al. (1999) also expressed staphylokinase in the expression system of *B.subtilis* [135] and Cheng et al. (1998) expressed this protein in *Streptomyces lividans* [138]. A review of other studies shows that Apte-Deshpande et al. (2009) obtained a recombinant *P. pastoris* strain GS115 including multiple inserts of the staphylokinase gene for high level expression about 1 g/L. The addition of antibiotics such as tunicamycin during the induction phase resulted in the expression of non-glycosylated r-sak of about 15 kDa. It was shown that prior to deglycosylation, the activity was lower than 2.5 mg U/mg but after the deglycosylation, this activity reached 95 U/mg [137]. Pulicherla et al. (2011) isolated the staphylokinase gene from the wound samples and the fibrinolytic activity was determined using a lysis test after *E. coli* salt-induced expression [152].

Miele et al. expressed staphylokinase in both *E. coli* and *P. pastoris* GS115. They showed that *Pichia* glycosylated staphylokinase in asparagine-28 position. The ratio of glycosylated to non-glycosylated forms was 5 to 6, separated by the cancavalin-A column. The SakSTAR variant was obtained highly functional at a concentration of 300–400 mg/L of purified protein from the expression system of *E.coli.* In addition, 60 mg/L of functional non-glycosylated SakSTAR and 50 mg/L of non-functional glycosylated one were also obtained from the *P. pastoris* strain GS115 [143].

Nguyen et al. (2012) observed the activity of 20658 U/mg after expression of SAKϕC variant in *P. pastoris* strain GS115 [30] while they expressed this staphylokinase variant with activity of 15175 U/mg in *E. coli* [131]. Staphylokinase expression in *Pichia* strain was reported in the 100% glycosylated form. After condition optimization, the temperature of 37 °C for the staphylokinase was found to be the optimum temperature in both *E. coli* and *P. pastoris*. Temperature stability of staphylokinase in both expression systems was 20–45 °C. The optimum pH of the staphylokinase expressed in *P. pastoris* and *E. coli* were 7 and 7.5 in phosphate buffer and,8 and 9 in the Tris buffer, respectively [30, 131].

Faraji et al. (2017), after codon optimization, expressed the SAKϕC in *P. pastoris* strains KM71H and GS115. The

Table 2 Overview of the expression levels and properties of staphylokinase or its multi-functional derivatives in various expression systems

References	Protein	Expression system	Yield (mg/Lbroth)	Ratio of glycosylated/non glycosylated forms	Characterization	Comments
[116]	rSAK	*Pichia pastoris*	Nearly 1 g/L	Mostly glycosylated	Thrombolytic activity of 2.5 U/mg for glycosylated form and 95 U/mg for non- glycosylated form achieved	Very high level expression due to multiple insertions of the SAK gene into *P. pastoris* genome with negligible plasminogen activation activity achieved. Plasminogen activation activity was measured by using S2251 as substrate
[125]	rSAK	*P. pastoris*	310 mg/L	Mostly glycosylated	Enzymatic activity was 21042 U/mg and 9002 U/mg for purified and supernatant proteins, respectively. Deglycosylation by using tunicamycin in culture medium, days 2 and 3 enhanced the activity to 43,858 U/mg	High level expression due to codon optimization achieved. Fibrinolytic activity was measured according to well fusion method with streptokinase as standard
[124]	rSAK	*P. pastoris*	33–50 mg/L	ratio of 5:6	50 mg/L of non- functional glycosylated form and 60 mg/L of functional non-glycosylated form achieved	Plasminogen activation activity was measured by using S2251 as substrate.
[124]	rSAK	*Escherichia coli*	300–400 mg/L (purified)	Only non-glycosylated form	Functional non -glycosylated	Plasminogen activation activity was measured by using S2251 as substrate
[109]	rSAK	*P. pastoris*	19 mg/L	glycosylated	Enzymatic activity of 20,658 U/mg of purified protein achieved	Plasminogen activation activity was measured by using AAS as substrate. Surprisingly, high activity of glycosylated form was reported
[114]	SAK-RGD-K2-Hirul	*P. pastoris*	8.21 mg/L	Not defined	SAK-RGD-K2-Hirul, in comparison with -SAK and SAK-RGD-K2-Hir, is a faster-acting and more potent form and it is better in terms of antithrombin and antiplatelet properties	Efficiency of blocking the platelet–platelet interaction by SAK-RGD-K2-Hirul was around 7% better than that of SAK-RGD-K2-Hir and comparable to that of the RGD sequence alone

Table 2 (continued)

References	Protein	Expression system	Yield (mg/Lbroth)	Ratio of glycosylated/non glycosylated forms	Characterization	Comments
[108]	Staphylokinase-Hirulog	*E.coli*	Quantity of the purified protein was 913 mg/L	Only non-glycosylated form	Compared to 21910 U/mL of SAK, the fibrinolytic activity of purified sak variant was 21825 U/mL with specific anti-thrombin activity of 1200 ATU/mg	Cold shock expression vector pCOLDI induced at the low temperature (15 °C) produced soluble protein
[134]	SAK- Thrombin recognition peptide-Hirudin (HV2)	*E.coli*	1.48 g/L	Only non-glycosylated form	Fed-batch fermentation in complex medium with feeding medium including glucose + magnesium sulfate, yeast extract and tryptone at 40 L fermenter produced fibrinolytic activity up to 1.53×10^5 IU/L	Optimum condition were successfully scaled up to 40 L fermenter.Fibrinolytic activities was determined on a fibrin plate by using SAK (1×10^4 IU/mg) as standard
[122]	rSAK	*E.coli*	2.8 g/L	Only non-glycosylated form	The purified SAK protein revealed plasminogen activation activity	–
[6]	SAK-RGD- Hirulog	*E.coli*	Quantity of the purified protein was 270 mg/L	Only non-glycosylated form	Very highly activity 102730 U/mg of SAK-RGD- Hirulog compared to102955 IU/mg of SAK achieved. The antithrombin and anti-aggregation activity of SAK-RGD-Hirulog was significantly higher than SAK	It was expressed in osmotically inducible *E. coli* GJ1158 as soluble form. Fibrinolytic activity was measured according to well fusion method with streptokinase as standard
[109]	rSAK	*E.coli*	300 mg/L	Only non-glycosylated form	15175 U/mg of purified protein achieved	Plasminogen activation activity was measured by using AAS as substrate
[120]	rSAK	*E.coli*	approximately 200 mg	Only non-glycosylated form	Intravenous administration of 10 mg rSAK over 30 min in five patients with acute myocardial infarction induced complete coronary artery recanalization, without associated fibrinogen degradation	Neutralizing antibodies was appeared in the plasma of all patients within 12 to 20 days

Table 2 (continued)

References	Protein	Expression system	Yield (mg/Lbroth)	Ratio of glycosylated/non glycosylated forms	Characterization	Comments
[121]	rSAK	*E.coli*	Quantity of the purified protein was 70–500 mg/L.	Only non-glycosylated form	The omission of 10 N-terminal amino acids had no effect on plasminogen activation. However, additional deletion of Lys11 led to the elimination of plasminogen activation	Substitution of Lys^{10} with His, Lys^{11} with His, Lys^{11} with Cys abolished plasminogen activation
[119]	rSAK	*E.coli*	15 mg/L into periplasm and 5 mg/L to extracellular media	Only non-glycosylated form	Expression, secretion and activity of rSAK was confirmed by the plate assay and plasminogen-coupled chromogenic substrate assay	–
[103]	PLATSAK (SAK-RGD-Hirudin-Fibrinopeptide A)	*E.coli*	10 mg scale (The standard purification experiment yielded about 1 mg per 200 mL culture)	Only non-glycosylated form	Despite slightly decrease in fibrinolytic activity compared to SAK, the purified fusion protein significantly lengthened aPTT and TT and inhibited the amidolytic activity of thrombin	Platelet aggregation was not markedly inhibited by PLATSAK, probably due to the unfavorable three dimensional structure, with the Arg-Gly-Asp sequence buried inside
[135]	rSAK	*E.coli, B. subtilis, Streptococcus sanguis*	10 mg scale	Only non-glycosylated form	In terms of serological surveys, the investigated SAK obtained from heterologous hosts was similar to that of authentic SAK obtained from *S. aureus*	The sak42D gene was expressed and secreted most efficiently by *B. subtilis* cells reduced in exoprotease production (25 mg SAK/L of culture supernatant)
[88]	rSAK	*B.subtilis*	100 mg scale	Only non-glycosylated form	The highest level of specific expression of SAK (mg/L/O.D.) in strain WB700 was 9.5 obtained at 200 rpm with O.D 6. Adding sucrose at O.D. 4 (100 g/h for 4 h) led to O.D 28.4 with SAK yield of 255 mg/L	The highest SAK yield using strain WB700 at 8 h after inoculation was113 mg/L provided at high cell density (OD 15.2) with agitation speed 400 RPM

Table 2 (continued)

References	Protein	Expression system	Yield (mg/Lbroth)	Ratio of glycosylated/non glycosylated forms	Characterization	Comments
[113]	rSAK	*B.subtilis*	337 mg/L	Only non-glycosylated form	The use of strain of protease deficient WB700 increased expression of staphylokinase without N- terminal heterogeneity, from the sucrose-inducible plasmid	–
[123]	rSAK	*E.coli*	Not defined	Only non-glycosylated form	The heated plasma agar plate test showed very good clearance zones after overnight incubation through induction. Incubation for 48 h led to leaky expression of SAK of un induced *E. coli*	The tube test showed nearly 70% residual clot weight within two hours of clot incubated with cell lysates
[91]	SAK-RGD-K2-Hir	*Schizosaccharomyces pombe*	Not defined	Not defined	A higher potential of SAK-RGD-K2-Hir was observed accompanied by faster and deeper lysis of 125I-labeled fibrin clots in human. The potency of thrombin inhibition by the hirudin part of the recombinant fusion protein SAK-RGD-K2-Hir was the same as that of r-Hir alone	Similar inhibition of platelet aggregation was detected for both SAK-RGD-K2-Hir and RGD at low concentrations whereas at high concentrations, the inhibitory effect was decreased for SAK-RGD-K2-Hir in comparison with RDG

Table 2 (continued)

References	Protein	Expression system	Yield (mg/Lbroth)	Ratio of glycosylated/non glycosylated forms	Characterization	Comments
[132]	RGD-hirudin	*P. pastoris*	About 1.5 g of purified RGD-hirudin was generated from 1 L culture	Only non-glycosylated form	Thrombin activity was over 12,000 ATU/mg. The expression product in the culture was up to 3500 ATU/mL. TT, PT, and aPTT (in the same way as wt- hirudin) were prolonged by RGD-hirudin, but just RGD-hirudin was able to inhibit the PAGm. According to histopathological analyses, RGD-hirudin, compared with wt-hirudin, had a greater effect (two or three times more than that of wt-hirudin) on the thrombosis prevention	Thrombin activity was determined by fibrinogen solidification assay
[127]	Mature sak - Hirulog	*E. coli*	10081 to 19928 U/mL	Only non-glycosylated form	Based on taguchi design and the further optimization using response surface methodology with 30 experiments, enzymatic activity increased highly by 1.32 folds (7580 to 10,081 U/mL) and yield by 1.98 folds (10081 to 19928 U/mL). Glucose, K2HPO4, TMM and KH2PO4 were the most influencing parameters	The significance of different factors of culture was revealed by Taguchi (7 factors including glucose, K2HPO4, TMM, KH2PO4, NH4Cl, yeast extract and $MgSO_4$)
[115]	SAK-RGD	*P. pastoris and E. coli*	Not defined	Only non-glycosylated form	The recombinant fusion protein showed an optimum temperature of 37 °C and it was stable in a temperature range of 30 to 42 °C. The expression of the SAK RGD from *P. pastoris* was the same expression as *E. coli*	Fibrinolytic activity was found according to well fusion method

Table 2 (continued)

References	Protein	Expression system	Yield (mg/Lbroth)	Ratio of glycosylated/non glycosylated forms	Characterization	Comments
[128]	Hirudin E.Coil- SAK K.Coil heterodimer	*B. subtilis*	50 mg/L (under the co-cultivation condition)	Only non-glycosylated form	HE-SAKK was capable of targeting thrombin-rich fibrin clots and inhibiting clot-bound thrombin activity comparable with their parent molecules	AS compared to SAK, HE-SAKK shortened 21 and 30%, respectively, time required for lysing 50% of fibrin clot in the absence or presence of fibrinogen. That also decreased at least 12 fold concentration required for 50% plasma clot lysis
[118]	SAK1, SAK2	*Hansenula polymorpha*	Nearly 1.1 g/L	Stronger smear-like band of glycosylated rSAK-1 and weaker non smear non-glycosylated one was appeared along with SAK-2 variant with only non-glycosylated band	Whereas enzymatic activity of SAK-1 reduced substantially, enzymatic activity of SAK2 was measured between 56.232 and 58.446, depending on production scale in fermenter	Enzymatic activity was measured by using SAK reference standard (SAK STAR 94/718)
[111]	rSAK	*E.coli*	Not defined	Only non-glycosylated form	Growth increased in Luria–Bertani agar containing IPTG in a concentration of 100 g/mL and incubated at 37 C° for 24 h. Diameter of zone of hydrolysis was 30 mm at 37 °C, while the diameter was decreased above and under this temperature	Enzymatic activity was found according to well fusion method

S2251 d-valyl-leucyl-lysine-p-nitroanilidedihydrochloride, *AAS* N-(p-tosyl)-gly-pro-lys 4-nitroanilide acetate salt

expression maximum reached up to 310 mg/L of the culture medium after 48-h stimulation with 3% methanol and remained steady until Day 5. The maximum activity of the enzyme was at pH 8.6 and 37 °C. It was highly active in the temperature range of 20–37 °C and pH range of 6.8–9. The specific activities of rSAK were measured as 9002 and 21,042U/mg for the non-purified and purified proteins respectively. Further, Deglycosylation by using tunicamycin in culture medium enhanced the activity to 43,858 U/mg. According to western blot analysis, a prominent band of about 22 kDa and a weaker band of 18.6 kDa was observed. This suggested that secreted proteins were mostly glycosylated [153].

Expression of multi-functional staphylokinase derivatives in different hosts

Several chimeric proteins with enhanced staphylokinase properties, which benefit from thrombin inhibitor and platelet aggregation inhibitor, were produced to prevent re-blockage of the vessels [6, 7, 112]. Different methods for optimizing the concentration of the contents of the medium also used to increase the production of staphylokinase variants with maximum fibrinolytic properties [154]. Van et al. (1997) designed PLATSAK with SAK linked to RGD as an anti-platelet in addition to hirudin and parts of fibrinopeptide A as thrombin inhibitor expressed in the *E. coli*. This combination showed a slight decrease in fibrinolytic activity; its anti-thrombin activity was high and anti-platelet activity was low [124]. In another study by Lian et al. (2003), staphylokinase and hirudin were linked through a pair of coiled-coil sequences acting as a heterodimer domain. A coiled-coil sequence enriched with lysine (K-coil) was added to the C-terminus of staphylokinase to produce SAK-K coil (SAKK). Continually, a coiled-coil sequence enriched with glutamic acid (*E. coli*) was added to the end of the C-terminal of the hirudin to produce hirudin-*E.coil* (HE). This heterodimer molecule (HE-SAKK) was a potent thrombolytic agent in comparison with staphylokinase confirmed by thrombin-rich fibrin and plasma clot lysis studies (under in vitro condition). As well as, this heterodimer could reduce clot reformation during fibrinolysis [155].

Chen et al. (2007) made a targeted mutagenesis in lysine 35 staphylokinase and replaced it with arginine. In this study, RGD-SAK showed a higher tendency to platelet than staphylokinase. The thrombolytic activity and ADP-induced platelet anti-aggregation showed a dose-dependent behavior [156].

SAK-RGD-K2-Hir and SAK-RGD-K2-Hirul were developed by Szemraj (2005) in yeast *Schizosaccharomyces pombe* and Kowalski (2009) in *P. pastoris*, respectively [7, 112]. In these structures, the kringle 2 domain of t-PA protein, which has a fibrin binding site, were added to staphylokinase along with RGD, hirudin or hirulog which caused a more fast-acting and higher potent thrombolytic agent in comparison with standard staphylokinase. Indeed, in addition to the activity of anti-thrombin and anti-platelet, the fibrinolytic activity also increased significantly in both fusion proteins compared to rSAK.

Wang et al. (2009) used only 12 amino acids of thrombin-binding domain of hirudin (HV) and expressed the SAK-HV variant in *E. coli* BL21 (DE3) [122]. In another similar study, a fusion protein including functional small domain of hirudin variant 1 (HV1) was added to both N- and C-terminal of staphylokinase to produce SAK-HV1 and HV1-SAK. In this study, the rate of plasminogen activation by SAK was not changed by the presence of an additional N- or C-terminal peptide sequence. However, cleavage at N-terminal lysines made the N-terminal fusion unstable against plasmin [157]. Therefore, C-terminal fusions created stable configurations for reasonable development of improved thrombolytic agents based on staphylokinase [157, 158].

Mo et al. (2009), fused hirudin and RGD along with SAK-hirudin expressed at high-level in *P. Pastoris* [159]. The anti-thrombin activity of the purified RGD-hirudin and SAK-hirudin were 12000 U/mg, which was equivalent to wild-type (wt), but only r-RGD-hirudin had an inhibitory effect on ADP-induced platelet aggregation. In animal models, SAK-hirudin and r-RGD-hirudin were three times more effective than wild type hirudin in preventing thrombosis. Animals injected with r-RGD-hirudin increased thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (aPTT), that were similar to wt-hirudin, but only r-RGD-hirudin was able to inhibit platelets aggregation rate maximum (PAGm) [159].

Pulicherla et al. (2012) also produced staphylokinase linked to RGD in methanol-induced *P. pastoris* strain GS115, which had a good thrombolytic activity [136]. After adding RGD motif using site-directed mutagenesis to staphylokinase, biochemical analysis revealed that RGD-SAK has a fibrinolytic activity similar to that of staphylokinase. RGD-SAK had a greater tendency to platelet binding under the in vitro condition than staphylokinase. A platelet-rich clot lysis test, in vitro, showed that RGD-SAK has a higher lysis property than staphylokinase. There was a significant reduction in the amount of concentration needed to obtain 50% platelet-rich clot lysis (C_{50}) in different concentrations of RGD-SAK in comparison with SAK. RGD-SAK inhibited ADP-induced platelet aggregation while staphylokinase had a negligible effect. In fact, RGD-SAK represented a bifunction which caused both lysis of platelet-rich clots and inhibition of platelets aggregation. On this basis, it was concluded that the addition of RGD may reduce vessel re-clotting [4]. Pulicherla et al. (2013) also expressed the chimeric staphylokinase (SAK-RGD-Hirulog) in salt-inducible *E. coli* strain (GJ1158). The fibrinolytic activity of the fusion type was

Table 3 Clinical trials based on staphylokinase

Study	Condition	Intervention	References	Status
Single bolus recombinant non-immunogenic staphylokinase (FORtelyzin) versus single bolus tenecteplase (Metalyse) in STEMI	Myocardial infarction	Drug: recombinant staphylokinase Drug: tenecteplase	NCT02301910	Unknown
Single bolus recombinant non-immunogenic staphylokinase (Fortelyzin) and bolus infusion alteplase in patients with AIS	Ischemic stroke	Drug: recombinant staphylokinase Drug: alteplase	NCT03151993	Recruiting
A trial using double-bolus THR-100 versus streptokinase (THR-100).	Acute myocardial infarction	Drug: THR-100 Drug: streptokinase	NCT01305226	Completed

very high, along with non-fusion one. SAK had a specific activity of 102,955 U/mg while the fusion type had a specific activity of 102,730 U/mg. There was no significant difference in the activity of these two proteins. Also, the fusion one showed significantly additional anti-thrombin and anti-platelet activities [6, 160].

Kotra et al. (2013) expressed staphylokinase with hirulog as a soluble (without inclusion body) at a high level (913 mg/mL) of *E. coli* expression system. In this study, a pCold1 vector that induces expression at 15 °C by IPTG was used instead of pET28a+. As a result, fibrinolytic activity of 21825 U/mL and the anti-thrombin activity of 1200 U/mL were obtained [130].

Clinical trials based on staphylokinase

In a the clinical trial in patients with coronary artery disease, Vander schueren et al. (1996) showed that the plasma half-life of staphylokinase was 6.3 min and double bolus administration was considered safe compared to single bolus administration [161]. Moreover, in other clinical trials conducted by Armstrong et al. (2000 and 2003), (Type 1 and 2 CAPTORS), the therapeutic potential of staphylokinase and its derivatives was studied and confirmed [162, 163]. However, Collen et al. reported the re-blocking of blood in vessels in 38% of patients treated with staphylokinase [164]. In order to treat MI, two new variants of staphylokinase (THR-100 and THR-174) are also suggested by ThromboGenics NV (Table 3). For this purpose, clinical trials (phase III) are conducted in India, the Middle East, Africa, and other countries [6].

Conclusions and perspectives

Thrombolytic therapy using current plasminogen activators is associated with complications that include: bleeding, fibrin specificity, and vessel re-blockage caused by the platelet-rich secondary clots. Therefore, the ongoing thrombolytic research has now focused on third-generation thrombolytic molecules with fewer side effects. Staphylokinase could be a promising thrombolytic agent with properties of cost-effective production and the least side effects due to highly fibrin specificity and resistance to effect of plasminogen activator inhibitor type I (PAI-1) released by active platelets. As well as for improved efficacy and safety profile, the effectiveness of therapeutic intervention of genetically engineered multi-functional staphylokinase is convincingly demonstrated. However, a drawback related to staphylokinase administration is the development of neutralizing antibodies against staphylokinase from the third week on in all patients. The plasma clearance and the immunogenicity of staphylokinase could be decreased by amino acid substitutions in addition to polyethylene glycol- derivatization of cysteine substitution variants. More dose-finding studies followed with randomized control trials will require to confirm the contribution of each modification to the overall effects.

Acknowledgements This study was supported by Molecular Medicine Research Center, Hormozgan Health Institute and the Isfahan Cardiovascular Research Center. We appreciated Dr.Mostafa Khedri for her valuable comments.

Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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