Evolutionary Trend of Thrombolytic – Their Significance

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Abstract

Thrombolytic drugs have been used in the clinical arena to treat a wide variety of venous and arterial thromboembolic disorders which are a main cause of death. Thrombolytic molecules are plasminogen activators that convert the inactive plasminogen to the active proteolytic enzyme plasmin which dissolves the blood clot. Fibrin specific plasminoactivators such as tissue – type plasmino activator (t-PA), single chain urokinase type PA, staphylokinase etc. are superior to the non-specific ones such as streptokinase and two chain urokinase-type-PA (tcu-PA). The main advantage of the latest generation of fibrinolytic drugs for treating acute myocardial infarction (MI) is their ease of administration. Four thrombolytic drugs are currently available in market: alteplase, reteplase, streptokinase and tenecteplase. Streptokinase became the most widely used drug due to its low cost. However, newer agents have a longer half-life, making them easier to deliver, but these also are associated with bleeding complications. In a search to overcome these drawbacks, many efforts have been made to develop the more potent thrombolytic agents without bleeding complications. In this review, different generations of thrombolytic agents have been discussed.

Keywords*: Thromboembolic disorders, plasminogen activators, Chimeric molecules, third*

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generation thrombolytics.

1. Introduction

Thrombotic diseases are responsible for heavy toll in death and disability worldwide. These are the most common diseases in the United States and in almost all Western Industrialized countries. Each year cardiovascular disease (CVD) causes 4.3 million deaths in Europe [1] while in United States 2.5 million deaths *i.e.* one of every 2.8 deaths [2]. Thromboembolic disorders, such as myocardial infaraction, cerebrovascular thrombosis, pulmonary embolism and venous thrombolism are life-threatening for human being. The imbalance between coagulation and fibrinolytic events results in the formation of thrombus as a blood clot where it partially block a vein or artery and also embolus which formed when these clot break off and travels through the bloodstream until it becomes lodged in a smaller blood vessel and leads in the blocking of blood supply [3].

Many clinical trials have conclusively shown the value of thrombolytics in the treatment of myocardial infarctions. For dissolving thrombus, the fibrinolytic system of patient has been activated by infusing plasminogen activators that convert inactive plasminogen to active plasmin which in turn digests fibrin to soluble degradation products [4]. These plasminogen activators (PA) are mainly categorized into two types: non fibrin specific PA such as streptokinase (SK) , two-chain urokinase-type PA (tcu-PA, urokinase), and another is fibrin specific PA such as tissue-type PA (t-PA) or single-chain urokinase PA (scu-PA) [5]. The fibrin specific drugs primarily accumulate onto the thrombus and expresses its plasminogen activity and results in an effective degradation of fibrin without systemic fibrinolytic state which shows its superiority than the non specific ones. In spite of superiority of fibrin specificity, these are also associated with some drawbacks. Even though tremendous efforts h**a**ve been made in the improvement of thrombolytic drugs for treatment of thromboembolic diseases, but much still remains to be done.

Table 1 . Desirable features of ideal thrombolytic drug

• Fibrin specificity • Good patency. • Low or no reocclusion rate and systemic bleeding • Resistant to plasminogen activator inhibitor-1(PAI-1) • Non antigenic and cost effective

2. Thrombolytic Agents

Thrombolytic agents are used to treat a wide variety of venous and arterial thromboembolic disorders; especially against acute myocardial infarction [6]. These are normally plasminoactivators that make use of the vascular system's own intrinsic thrombo resistance defense mechanism by accelerating and amplifying the conversion of an inactive precursor, plasminogen to the active enzyme plasmin, a natural fibrinolytic agent, which in turn hydrolyzes several key bonds in the fibrin (clot) matrix causing dissolution. These thrombolytic drugs would have ideally rapid reperfusion which can establish TIMI grade 3

flow in patients [7, 8]. The agent would also have prolonged half-life that permits single-bolus dosing, facilitating more timely and easy administration. Some of the desirable features of ideal thrombolytic agent are summarized in Table 1.

Thrombolytic agents can be categorized in several ways. Classification schemes can be devised on the basis of the source of the agent, the propensity for enhanced enzymatic activity on a fibrin or cell surface or the mechanism of action (enzymatic vs. non-enzymatic) or different generation wise. Each of these methods of classification is useful in helping to characterize the diverse nature of plasminogen activators, but regardless of how one defines these agents, they all serve one primary purpose *i.e* the conversion of plasminogen to plasmin. Each of these agents has been briefly discussed below (Table 2).

2.1 The first generation thrombolytic molecules

Initial studies on thrombolytic therapy were primarily focused on whether the treatment was effective in reducing mortality, on dosing regimens, and on the route of administration. Though the thrombolytic therapy for acute MI was first attempted in the 1950s, significant advances were not made until the 1980s.

2.1.1. Streptokinase: Streptokinase (SK), the oldest and best-known plasminogen activator, is produced by various strains of β-hemolytic Streptococci. Its fibrinolytic activity was first described in 1933 [9]. Streptokinase found its initial clinical application in combating fibrinous pleural exudates, hemothorax and tuberculous meningitis. In 1958, streptokinase was first used in patients with acute myocardial infarction and this changed the focus of treatment. Earlier streptokinase produced contradictory results until the GISSI (Gruppo Italiano per la Sperimentazione della Streptochinasi nell'Infarto Miocardico) trial in 1986, which validated streptokinase as an effective therapy [10].

Table 2. Generational classifications of the thrombolytic Agents

Generation of Thrombolytic Drug	Fibrin Specific	Non Fibrin Specific
First	--	Urokinase *
	--	Streptokinase *
Second	Recombinant tissue plasminogen activator * (t-PA)	Pro-urokinase (scum-PA)
	Alteplase	Sk-plasminogen activating complex * (APSAC)
Third	Tenecteplase * (TNK-tPA)	--
	Reteplase *	--
	Monteplase	--
	Lanoteplase	--
	Pamiteplase	--
	Staphylokinase	--
	Desmoteplase (Bat-PA)	--
	Chimeric thrombolytics	--
* Approved for clinical use		

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Streptokinase is a non-fibrin specific extracellular enzyme that exerts its fibrinolytic action indirectly by activating the circulatory plasminogen. It has a molar weight of 47 kDa and is made up of single-chain polypeptide of 414 amino acid residues [11]. Streptokinase composed of three distinct domains, denoted as α (residues 1–150), β (residues 151–287), and γ (residues 288–414). Streptokinase is not an enzyme and therefore does not exhibit plasmin activity by proteolytic cleavage of plasminogen. Instead, it binds noncovalently to plasminogen in a 1:1 equimolar fashion and thereby confers plasmin activity. The streptokinase-plasminogen complex then acts on other plasminogen molecules to cleave the Arg -Val 561 bond and, as a result, generate plasmin [12]. SK has a plasma half-life of 30 minutes.

Streptokinase is available in the market as Streptase® and Kabikinase. Since it is a bacterial product, its use is normally associated with allergic reactions and antibody-mediated inhibition of plasminogen activation. SK is also associated with bleeding complications due to its non fibrin specificity.

2.1.2. Urokinase: Urokinase, also called urokinase-type Plasminogen Activator (u-PA), was originally produced from human urine. It is secreted as a 54kDa single-chain molecule (scu-PA, prourokinase) which can be converted to a two-chain form (tcu-PA) due to proteolytic cleavage at Lys^{158}-Ile^{159} by plasmin [13]. Urokinase (u-PA) a serine proteinase of 411 amino acid residues , consist of three domains: the carboxyl-terminal serine protease domain, the kringle domain and amino terminal the growth factor domain [14]. The two chain tcu-PA has full enzymatic activity but does not exhibit fibrin affinity while scu-PA has a weak fibrin affinity. 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 induces fibrin specific clot lysis. The initial half-life of scu-PA was 6-9 minutes upon administration to the patients with acute myocardial infarction.

Earlier Urokinase was available in the market in the brand name Abbokinase®, Kinlytic™ (Microbix Biosystem). It was obtained from human neonatal kidney cells grown in tissue culture.This drugs had been given as intra venously .Side effects was also associated with this as in case of streptokinase drug. In Dec 1998, the FDA removed the Abbokinase® (Abbort Laboratory, Abbott Park, Illions) from the market place due to significant deviation from current good manufacturing process. This has prompted to consider reteplase and alteplase as an alternative.

2.2 Second generation thrombolytic molecules

The search has continued for the ideal thrombolytic therapy .The need for a safer, more effective fibrinolytic drug led to the development of the second generation agents.

2.2.1 Saruplase: Recombinant scu-PA(r-proUK) is known as saruplase which is expressed in *E.coli* and obtained as 45kDa non glycosyalted molecule(21). Its therapeutic potential was investigated in several clinical studies in patients with AMI viz. PRIMI [22] SESAM [23] LIMITS [24] PATENT [25] COMPASS [26] or with stroke PROACT I and II (27, 28). Further, safety and efficacy of Saruplase was confirmed in the PASS study [29]. However, the U.S. Food and Drug Agency (FDA) has not approved r-proUK for clinical use and has demanded a new separate study.

2.2.2 **Anistreplase:** Anistreplase or anisoylated plasminogen-SK activator complex (APSAC) is an another form of SK,an equimolar acylated complex of human lys-plasminogen and SK.This complex act on plasminogen upon deacylation spontaneously in plasma. Activation of anistreplase to lys-plasminogen -streptokinase occurs by a first order, hydrolytic process, in blood or in the thrombus, with release of the p-anisoyl group by deacylation. The non-enzymatic deacylation process starts immediately after intra venous administration of anistreplase. Since it is a complex, it converts only those molecules of plasminogen to which the streptokinase is bound (it has no effect on endogenous plasminogen) [30]. The plasma half-life of APSAC is 70-120 min. The International Study of Infarct Survival (ISIS)-3 showed a 10.5% mortality rate at 35 days with anistreplase and an intracranial hemorrhage rate of 0.6% [25]. Eminase® (Wulfing Pharma GmbH) is the brand name of Anistreplase in the market. It has same side effects as the streptokinase has, but has the advantage of single-bolus administration.

2.2.3 Alteplase: Tissue plasminogen activator (tPA), a serine protease, is a fibrin specific thrombolytic molecule. It is synthesized as a single chain polypeptide of 70 kDa consisting of 527 amino acids [26]. Proteolytic cleavage of the Arg 275-Ile 276 bond by Plasmin, kallikrein, or factor Xa converts this single chain form (sct-PA) into a two-chain species (tct-PA). t-PA exhibit its enzymatic activity both in single chain and two chain form [27].The t-PA molecule (two chain form) contains four domains; a fibronectin type I finger (F) domain with NH_2-terminal region of 47-residues (residue 4 to 50) , an epidermal growth factor domain (residue 50 to 87) and two kringle domains comprising residues 87 to 176 and 176 to 262 (K1 and K2) and a serine proteinase domain (residues 276 to 527) with the active site residues His ^{322}, Asp ^{371} and Ser ^{478} [28].

Structure function analysis has revealed specific functions for the t-PA domains. The stimulation of t-PA activity by fibrin and also its high fibrin affinity are mediated by the finger and kringle 2 domains. The epidermal growth factor and carbohydrate side chains play an important role in its elimination from the circulation (29). Plasminogen activator inhibitor-1(PAI-1) inhibits the PA activity of t-PA [30]. The amino acid region at position 296-299 in the protease domain of t-PA is associated with the inhibition by PAI-1 [31]. t-PA is a poor enzyme in the absence of fibrin, but the presence of fibrin strikingly enhances the activation rate of plasminogen .Optimal stimulation of t-PA is only obtained after early plasmin cleavage in the COOH-terminal α-chain and NH_2-terminal β-chain of fibrin. 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 which results in its enhanced affinity for plasminogen activation [32]. 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 thus only slowly inactivated by α_2–antiplasmin. These molecular interactions mediate the fibrin specificity of t-PA.

Activase® (Alteplase) is a tissue plasminogen activator produced by recombinant DNA technology. It is synthesized using the cDNA for natural human tissue-type plasminogen activator obtained from a human melanoma cell line [33]. The clinical trials, COOL 1 and COOL 2, demonstrated the efficacy and safety of t-PA in the treatment of central venous access device (CVAD) occlusion [34]. Alteplase produces TIMI grade 3 flow in 50% to 60% of patients at 90 minutes. In GUSTO-1, patients who received alteplase had a 6.3% mortality rate at 30 days and a 0.72% incidence of intracranial hemorrhage [35]. Compared with streptokinase, alteplase resulted in a 1% absolute reduction in death or

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nonfatal stroke. Activase® (GENENTECH) is a sterile, white to off-white, lyophilized powder for intravenous administration after reconstitution with Sterile Water for Injection. This medication is given by injection directly into the vein within 3 hours after the onset of stroke symptoms. The most common and serious side effect with alteplase is bleeding.

2.3 Third generation thrombolytic molecules

The third-generation thrombolytic agents were developed to improve the efficacy, safety, and ease of administration. The major thrust has been on modifications of rt-PA to develop a drug that restores faster patency and more effective manner as well as being easier to administer .These newer agents are more fibrin specific, that bind to thrombus and predominantly activate clot-bound plasminogen, thus avoiding systemic plasminogen activation. Due to their longer half-life, the newer drugs can often be administered in a single bolus or two boluses.

2.3.1 Reteplase: Reteplase (recombinant plasminogen activator, r-PA) is a single chain deletion variant of alteplase that is expressed in *Escherichia coli*. It consist of amino acids1-3 and 176-527(deletion of Val 4-Glu175): the Arg275-Ile276 plasmin cleavage site is maintained. Reteplase, lacking fibronectin finger region and epidermal growth factor domain, consist of only kringle 2 and protease domain of the t-PA. It is a non glycosylated protein of 355 amino acid with 39k Da. It has similar plasminogen activator activity as alteplase in the absence of fibrin,but its binding affinity to fibrin is significantly (5 fold) lower than that of alteplase due to deletion of the fibronectin finger region. Although kringle 2 domain, which is known to stimulate protease in the presence of fibrin, is part of the reteplase molecule, it is stimulated in the presence of fibrin to a lower extent than alteplase, suggesting that the fibronectin finger is involved in the stimulation of the protease as well [36]. Reteplase and alteplase do not differ with respect to their inhibition by the plasminogen activator inhibitor type 1 (PAI-1). Due to deletion of the finger domain, the epidermal growth factor domain and kringle 1domain as well as carbohydrate side chain, the hepatic clearance of reteplase is significantly reduced. Consequently, plasma half life is increased to 14–18 minutes against the alteplase half life of 3–4 minutes. This allows reteplase to be administered as boli against as an initial bolus followed by an infusion, as in case of alteplase.

The efficiency of reteplase in patients with acute myocardial infarction has been evaluated. In the RAPID I (recombinant plasminogen activator angiographic phase II international dose finding study) trial, where reteplase was administrated as a double bolus (10 plus 10 MU 30 min apart) against the standard dose of alteplase (100 mg over three hours), the patency of reteplase was reached earlier and more frequently than with alteplase. This was confirmed in the RAPID II (reteplase vs alteplase patency investigation during myocardial infarction) trial where the same reteplase dose regimen revealed a higher rates of early reperfusion than accelerated regimen of alteplase (over 1.5 h) [37]. Furthermore, in the GUSTO (global use of strategies to open occluded coronary arteries) III trial mortality after 30 days of patients treated with reteplase was not significantly different from that with alteplase and those treated with alteplase which led to conclusion that reteplase was superior to anteplase in its administration as a double bolus [38]. However bleeding and allergic reactions are also associated by using this drug.

2.3.2 Tenecteplase: Tenecteplase is a multiple (three) point mutation of alteplase, rather than a deletion mutant (as reteplase does). It has been specifically bioengineered to have an

extended half-life, allowing convenient single bolus dosing, increased fibrin specificity and patency. The substitutions of Asn ^{117} with Gln delete the glycosylation site in kringle 1 domain while the replacement of Thr^{103} with Asn reintroduced a new glycosylation site, but at different locus. These modifications prolong its half life (17-20 min.) by reduction in plasma clearance rate. The amino acids Lys ^{296}-His^{297}-Arg^{298} -Arg^{299} are each replaced with Ala which increases resistance by 80 times to inhibition by PA-1 [40]. Tenecteplase has a similar ability as native t-PA to bind to fibrin with enhanced fibrin specificity 14-fold greater [41].

The efficacy of tenecteplase has been evaluated in several clinical studies. It does not produce the paradoxical procoagulant effect that limits other thrombolytic agents. The long half life (17-20 min.) allows for single bolus administration with 6 to 12 fold greater thrombolytic patency than accelerated tissue type plasminogen activator on a $\mu g.kg^{-1}$ basis. It has faster reperfusion, faster clot lysis, longer duration of arterial patency, and lower occurance of bleeding compared with tissue type plasminogen activator [43]. In the ASSENT-1 (assessment of safety and efficacy of a new thrombolytic agent) trial in patients with acute myocardial infarction, single bolus tenecteplase proved to be as safe as the gold standard of thrombolytic therapy, the accelerated regimen of alteplase (initial bolus followed by an infusion over 90 minutes) [44]. In the TIMI-10B (thrombolysis in myocardial infarction) trial single bolus administration of 40 mg tenecteplase revealed the same rate of patency at 90 minutes after the initiation of thrombolytic therapy as accelerated regimen of alteplase [45]. Furthermore the phase III ASSENT-2 trial tenecteplase and alteplase were equal with respect to total mortality after 30 days. As with reteplase, tenecteplase in comparison with alteplase is equal in efficacy and superior in its application as a single bolus [46]. Bleeding and allergic reactions are also associated by using this drug.

2.3.3 Lantoplase: Lantoplase is a deletion and single point mution of wild-type t-PA. It lacks fibronectin finger region and the epidermal growth factor domain. In addition, due to substitution of Asn ^{117} with Gln, the glycosylation site in kringle 1 domain is deleted. Since this carbohydrate chain is linked with hepatic elimination, its removal prolongs the plasma half life of lanoteplase , that is about 10 times in comparison with alteplase [47].

The long half-life of lantoplase (37 min) allows for single bolus administration in the thrombolytic therapy. In the InTIME-1 (intravenous t-PA for treatment of infarcting myocardium early) trial, treatment with 120 kU/kg of lanoteplase resulted in a higher patency rate of the infarct related coronary artery at 90 minutes than treatment using alteplase in the accelerated regimen [48]. However, in InTIME-2 trial, it was found that both lantoplase and alteplase have similar efficiency with respect to overall mortality at 30 days. However, the rate of haemorrhagic stroke with lantoplase (1.12%) was significantly higher than that with alteplase (0.64%, $p = 0.004$) [49]. This increase in the most severe complication of thrombolytic therapy has stopped lantoplase from entering the market to date.

2.3.4 Monteplase: Monteplase is single amino acid point mutant of t-PA. The Cys 84 in the epidermal growth factor has been replaced by Ser [50]. The half life of monoteplase is longer than alteplase (23 min vs. 4 min), allowing a single bolus administration of .22mg/kg [51]. COMMA trial revealed that monteplase could be used independently of PA-1 kinetics [52]. However, thrombolysis with monteplase, in combination with catheter-based treatment, is an effective and safe therapy and has been approved for acute major pulmonary embolism (PE) in Japan [53].

2.3.5. Pamitelase: Pamitelase is a derivative of t-PA recombinant Chinese hamster ovary cell

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(CHO) lines. The kringle domain is deleted and there is a substitution of Arg at position 274 at the cleavage site of single chain t-PA which provide resistance to conversion into two chain molecule by plasmin [54]. The longer half-life and increased potency with distinct fibrin specificity were evaluated in a clinically relevant model of embolic stroke. A pharmacokinetic study in human being revealed a half life of 30-47 min administration of a single dose of 0.5 mg to 4 mg pamitelase [55]. Furthermore, the thrombolytic effect of a bolus administration of pamitelase was found to be similar with that of wild t-PA [56].

2.3.6. Desmoteplase: Desmoteplase is a plasminogen activator derived from the saliva of the vampire bat *Desmodus rotundus*. It exists as a single chain molecule of 447 amino acids. Its structure is similar to u-PA, consisting of one kringle domain with catalytic domain. It has been produced recombinantly [57]. Desmoteplase is more fibrin dependent and fibrin specific than t-PA. Its catalytic efficiency increases thousands times in the presence of fibrin while t-PA is enhanced by only 72 fold [58]. The extended half life of desmoteplase (2.8h in a phase I clinical study) suggest that this agent would be appropriate for single bolus dosing. Preliminary studies of the DIAS trial (Desmotepase in Acute Stroke) also suggest the single bolus of 0.125mg/kg is the effective dose. It promoted faster and more sustained reperfusion than human t-PA without systemic plasminogen activation in a canine model of arterial thrombosis [59]. A lower degree of neurotoxicity as compared to rt-PA has been suggested in animal model [60]. Therefore, it is of interest to apply the desmoteplase to the stroke patients.

2.3.7. Staphylokinase (SAK): Staphylokinase (SAK) is a fibrin specific clot dissolving agent for myocardial infraction.It is an extracellular protein secreted by *Staphylococcus aureus* strain after lysogenic conversion with bacteriophages. Matured staphylokinse consists of a single polypeptide chain of 136 amino acids without disulfide bridges. Its molecular weight is approximately 16kDa [61]. Its mechanism of activation bears similarities to that of streptokinase along with some differences. Like streptokinase, it is not an enzyme and forms a 1:1 stoichiometric complex with plasminogen but unlike the streptokinase-plasminogen complex , the staphylokinase-plasmin complex is inactive and requires for conversion to staphylokinase:Plasmin complex to expose their active site and become a potent plasminogen activator ($Km=7\mu mol/L, k_{cat}=1.5s^{-1}$) [62].

The thrombolytic properties of staphylokinase have been evaluated in several clinical studies. It is highly fibrin specific but lesser fibrinogenolytic properties as compared to streptokinase. In the STAR trail randomnised 100 patients were received either of two staphylokinase doses (10 mg or 20 mg) or accelerated t-PA. The staphylokinase groups received 10% of the dose in an initial bolus, with the remainder of the dose administrated in a 30 min infusion. Systemic fibrinogen degradation, α_2-antiplasmin consumption, and plasminogen activation were substantial with t-PA, but absent with staphylokinase. This observation confirmed that staphylokinse is highly fibrin specific [63]. Since the initial plasma half life of staphylokinase (6.3 min phase I trial) does not support single bolus administration and a multicentre, randomized trial was done for demonstration of the feasibility and safety of a double-bolus administration [64]. The staphylokinase group received double bolus of 15 mg each, given 30 min apart. TIMI grade 3 flow was achieved in 68% of the staphylokinase group and 57% of t-PA [65].

The therapeutic potential of SAK and its derivatives has been studied in the CAPTORS (Collaborative Angiographic Patency Trial of Recombinant Staphylokinase) I and II trials that support its therapeutic potential [66, 67]. Although clinical experiences with staphylokinase has been encouraging, but limited. Being a heterologus protein,

staphylokinase is immunogenic in man who induces antibody formation and resistance to repeated administration [68]. Furthermore, its potency is also limited by incapability of mediating early reperfusion in 38% of treated patients and by rethrombosis [69]. In order to reduce immunogenicity and to prolong the half-life of SAK, a comprehensive site directed mutagenesis program had been done without the substantial loss of their fibrinolytic potency and fibrin specificity. The polyethylene glycol-derivatized cystein-substitution variants of recombinant SAK (Code SY161-P5) were generated. Furthermore, SY 161 with Ser in position 3 mutated into Cys, which was specifically PEGylated with maleimide-PEG (P) with molecular weight 5kDa (P5), 10,000(P10) or 20,000(P20), and characterized *in vivo* and *in vitro* [70, 71].

3. Chimeric Thrombolytic Agents

Now a day's several recombinant chimeric fibrinolytic agents are coming with more efficacies. They are either conjugates of plasminogen activators with monoclonal antibodies against fibrin, platelets, or thrombomodulin or hybrids of different plasminogen activator. There are lot of efforts have been done for selection of most potent combination of antibody and effector plasminogen activator agents for recombinant production. Prourokinase and staphylokinase chimera are most effective conjugates with monoclonal antibodies.

3.1 Staphylokinase chimeric molecule

Staphylokinse chimera are created by its cross linkage with and hirudin to antifibrin and antiplatelet antibodies .Now a days, there is an evidence that activated platelets play a pivotal role in arterial thrombosis and rethrombosis. The platelets aggregation is closely involved in the reformed secondary clots after thrombolytic therapy [72]. Arg-Gly-Asp (RGD) peptide can recognize the platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa) receptor. It has been also found that the binding of surface glycoprotein GPIIb/IIIa to fibrinogen mediates platelet aggregation and RGD can prevent fibrinogen binding to GPIIb/IIIa on activated paltelets, thus inhibiting paltelets aggregation [73]. Hence, it is interesting to hypothesize that the clot lysis efficacy of SAK can be enhanced with direct active platelet binding ability and at the same time the rethrombosis complication can be minimized with antiplatelet aggregation.

In effort to combine the advantages of staphylokinase with those of thrombin inhibitor and Platelet aggregation inhibitor, for preventing reocculusion, several chimeric proteins have been produced. Hirudin was selected as antithrombin partner due to its compatibility with the secretion system. In one approach, a model fusion protein constructed by joining to the small functional domain hirudin variant (HV1) in both N- and C- terminal configuration, HV1-SAK and SAK-HVI. The rate of plasminogen activation by SAK was not altered by the presence of an additional N- and C- terminal protein sequence. However, cleavage at N- terminal lysines within SAK rendered the N- terminal fusion unstable. Therefore, C- terminal fusions represent stable configuration for rational development of improved thrombolytic agents based on SAK [74].

In an approach, SAK and hirudin were joined together via a pair of engineered coiled sequences that act as the hetero dimerization domain. A lysine rich coiled coil sequence (K-coil) is added to the C-terminal tail of SAK to generate SAK-K coil (SAKK) and a glutamate rich coiled coil sequence (*E. coil*) is added to the C-terminal end of hirudin to generate

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hirudin-*E. Coli* (HE). These heterodimeric molecule (HE-SAKK) is a superior thrombolytic agent in comparison to staphylokinase as confirmed by *in vitro* fibrin and plasma clot lysis studies [75]. The acquisition of RGD sequence is done by site directed mutagenesis in which K^{35} is substituted with Arg and results in novel SAK variant RGD-SAK which had a much higher affinity with platelets than SAK and thus enriching the thrombolytic avtivity. It also inhibits ADP-induced platelet aggregationin in a dose dependent manner [76]. PLATSAK(Platelet-Anti-thrombin-Staphylokinase) was designed which include three inhibitory regions RGD sequence, a part of fibrinopeptide A, an inhibitor of thrombin and the C- terminal of hirudin a direct antithrombin [77]. In an another approach of improvement of the thrombolytic potential of SAK,as well as to introduction of antithrombotic and antiplatelet activity, the recombinant SAK-RGD-K2-Hir was constructed which is more potent and fast acting thrombolytic agent as compared with standard r-SAK [78,79].

3.2. Prourokinase chimera

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Single-chain u-PA (scu-PA) does not bind to fibrin which limits its thrombolytic potential.Targeting of scu-PA to a thrombus by combining the fibrin-specific antibody with its catalytic domain in a single molecule has resulted in a significant increase of both its *in vitro* fibrinolytic and its *in vivo* thrombolytic potential. This chimera construction has been achieved by chemical conjugation of scu-PA and a monoclonal antibody directed against the NH_2-terminal region of the β-chain of fibrin, 59D8 (80) or a monoclonal antibody specific for fragment D-dimer of cross-linked human fibrin (MA-15C5) [81]. A recombinant chimeric PA, rscu-PA-32k/59D8, in which intact 59D8 was fused to scu-PA-32k, yielded a 20-fold higher thrombolytic potency than scu-PA in a rabbit jugular vein thrombosis model [82]. Another recombinant chimeric PA, rscu-PA-32k/MA-15C5, composed of a scu-PA-32k and intact, humanized MA-15C5 (MA- I5C5Hu), yielded an 11 fold higher thrombolytic potency than scu-PA in the same model [83].

In one study a single-chain variable region antigen-binding fragment (scFv) derived from MA-15C5, scFv-KI2G_o that bound to fragment D-dimer of human cross linked fibrin with an affinity similar to that of intact MA- 15C5. The scFv-K12Go fragment was linked to rscu-PA- 33k, yielding a single-chain chimeric PA $K_{12}G_0S_{32}$, with a 13-fold higher *in vitro* fibrinolytic potency than rscu-PA-32k [84]. An another prourokinase chimera, scFv/lmw-scuPA, was designed by fusing lower molecular weight single-chain prourokinase plasminogen activator (lmw-scuPA) with a single-chain variable fragment (scFv) of a PECAM-1 antibody [85]. This fusion protein specifically targets endothelial cells *in vitro* and *in vivo* and provides antigen-specific enhancement of fibrinolytic activity in a mouse model of pulmonary thrombosis, providing evidence that vascular immuno targeting can be used for therapeutic fibrinolysis. Furthermore, since platelets play a key role when initially reperfused vessels reocclude, hence a monoclonal antibody 7E3 (blocks the platelet membrane glycoprotein (GP) lIb/Illa) has been conjugated with urokinase for construction of urokinase-7E3 Fab' chimera which reduces the rate of reocclusion and enhances the speed and efficacy of reperfusion [86].

3.3. Chimeric t-PA/scu-PA

This chimera plasminogen activator molecule consist of NH_2-terminal 1-263 amino acids of t-PA, which contain the structures responsible for its fibrin affinity, fused to the

COOH -terminal 144-411 amino acids of u-PA, which contain all structures required for the enzymatic and biological properties, characteristic of the intact molecule [87]. But it has reduced fibrin affinity as compared with t-PA due to steric interactions between the closely spaced functional domains of the two molecules. Therefore, to overcome this problem, an extended chimeric t-PA/scu-PA protein had been constructed, consisting of amino acids 1-274 of t-PA and 138-411 of Scu-PA, which has an additional sequence of 17 residues in the region joining the two proteins. The chimeric molecule thus contains the finger domain, the growth factor domain, and both kringle domains of t-PA, with additional sequence up to amino acid Phe^{274} which precedes the Arg^{275}-Ile^{276} peptide bond cleaved by plasmin. This region of t-PA is fused to the serine protease part of u-PA starting at Ser^{138}, 21 residues NH_2-terminal to the Lys^{158}-Ile^{159} peptide bond in scu-PA which is cleaved by plasmin [88].

Table 3. Comparison of different thrombolytic agents

Features → Thrombolytics ↓	Molecular weight (kD)	Immunogenicity	Plasma half-life (min)	Dose	Plasma Clearance (ml/min)	Plasminogen Activation	Fibrin specificity	Patency rate (TIMI-3 flow)	Development phase
Streptokinase	47	No	18	1.5 mU/hr	10.8 ±8.8	Indirect	(-)	30% at 90 min	Stroke-Pre registration studies MI-Approved
Anistreplase	131		90-112	?	65±25	Indirect	(-)	50% at 90 min	MI-Approved
Urokinase	32-54		15	3mU/hr		Direct	(-)	-	MI-Approved
Saruplase	47	No	6-9	20 mg bolus + 60 mg/60min	594±160	Direct	(-)	71.8% at 60 min	Stroke-proof of concept
Alteplase	70	No	4-8	15mg bolus+3hrinfusion upto	572±132	Direct	(+)	46-75% at 90 min	Stroke-Approved MI-Approved

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				85 mg					
Reteplase	40	No	11-14	Double bolus (10U + 10U, 30 min apart)	103±138	Direct	(+)	60-63% at 90 min	Stroke-proof of concept MI-Approved
Tenecteplase	70	No	20	0.5mg/kg single bolus	105	Direct	(++)	63% at 90 min	Stroke-preclinical MI-Approved
Lantoteplase	54	?	37+11	120, kU/kg single bolus	51±16	Direct	(-)	57-83% at 90 min	MI-Proof of concept
Monteplase	?	?	23	0.22 mg/kg single bolus	?	Direct	(-)	53-69% at 60 min	Preclinical
Pamiteplase	?	?	30-47	0.1mg/kg single bolus	135±10	Direct		50-54 % at 60 min	Preclinical
Desmoteplase	52	Yes	190	0.125mg/kg single bolus	23±62	Direct	(+++)	?	Stroke-Pre-registration studies
Staphylokinase	16.5	Yes	6	15 mg + 15 mg double	270±100	Indirect	(+++)	68% at 90 min	Stroke-Preclinical MI-Pre-registr

				bolu s					ation studies

Source . Current Vascular Pharmacology, 2006, Vol 4, No-1

This chimera showed markedly increased thrombolytic potency when compared with both parent molecules in different animal models. However, these findings were mainly due to a prolonged half-life [89]. Results of a clinical feasibility study in six patients with AMI suggest that administration of two 10 mg boluses of this agent can induce rapid, specific thrombolysis [90]. But it remains to be established to what extent these properties may be clinically useful.

4. Conclusion

Although currently clinically approved thrombolytic therapies have markedly reduced mortality following acute myocardial infarction and is gaining increasing acceptance for the treatment of various other thromboembolic disorders, all these drugs encompasses significant drawbacks, including the need of large therapeutic doses, limited fibrin-specificity and significant associated bleeding tendency and reocclution. Adjuvant drugs, which are generally given along with these thromolyic treatments, have an own side effects. Half or more of the patients will fail to achieve early and complete reperfusion with the current regimens.

To address these problems, many efforts have been made for its solution. Recent research has focused on the development of newer agents with features that may translate into improved efficacy and safety profile. The new, genetically engineered 'third generation' thrombolytic agents offer not only the promise of improved clinical outcomes but also the opportunity to determine the relative importance of fibrin specificity, plasma half-life, and resistance to inhibition by plasma inhibitors in thrombolytic therapy. In addition to increased efficacy, safety may also be enhanced especially when highly selective, antibody targeted plasminogen activators and antithrombin agents are used. However, the optimal thrombolytic strategy has yet to be determined which have the ability of 100% reperfusion and without bleeding and reocculusion complications. Further research will be expected to develop the perfect thrombolytic agents.

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