

## Staphylokinase: A Potent Thrombolytic Agent

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**Abstract:** As the incidences of Cardio Vascular Diseases (CVDs) is increasing all over the world, the deaths associated with CVDs have also, been on the rise. WHO estimates a total of 17.3 million deaths due to CVD every year which is set to rise to 24 million by 2030. Myocardial Infarction (MI) is one of the deadliest among CVDs group. An alarming number of people are falling prey to MI and most of them need a quick clinical intervention. Out of the many surgical and chemical treatments under development, thrombolytic enzymes have a huge potential and a major role to play. Staphylokinase (SAK) is one such enzyme that has the potential to be used as a thrombolytic agent. SAK is small, less immunogenic and doesn't cause systemic bleeding. This review discusses the basic aspects of thrombosis, CVDs, mechanism and the role of Staphylokinase to be used as a potent thrombolytic agent.

**Keywords:** Staphylokinase, Myocardial Infarction, Thrombolysis, Plasmin

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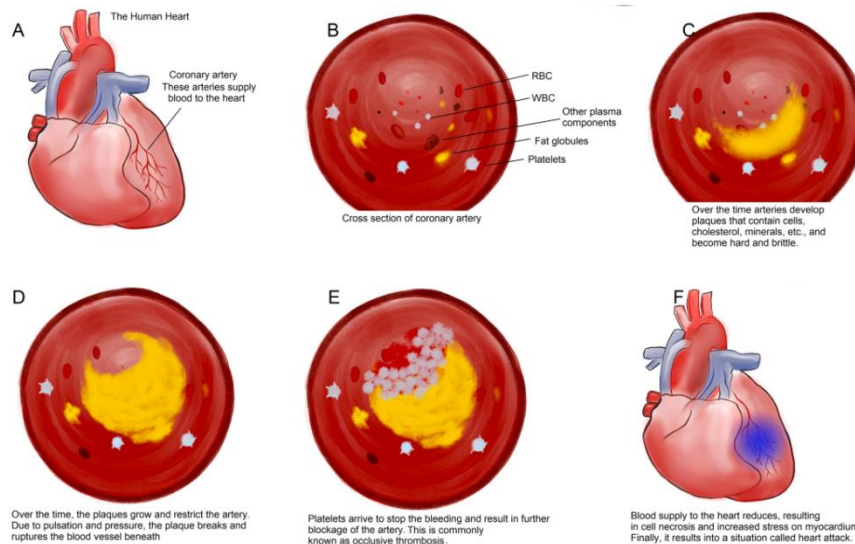
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### I. Introduction

Out of the total global 57 million deaths, non-communicable diseases claimed 36 million (63%) deaths. 17.3 million (30%) deaths alone occurred due to Cardio Vascular Diseases (CVDs). CVDs particularly heart attack and strokes are increasing exponentially and have become a leading cause of mortality and morbidity all over the world. As per the reports from the World Health Organization (WHO), at least 17.3 million people die every year, mounting to 31% of total deaths globally. Over 82% of deaths are reported from middle – and low – income countries (WHO fact sheet, May 2017, [1]). Deaths due to CVD are estimated to rise to 20.5 and 24.2 million by the year 2020 and 2030 respectively. Considering the proportion, deaths due to CVDs will rise to 32.5% by 2030. Undoubtedly, heart diseases are the major killers' world over. Heart diseases comprise several ailments; namely high blood pressure, congestive heart failure, stroke, Coronary Heart Disease (CHD), Rheumatic Heart Disease, etc. The deaths due to CVD are similar with 46% in males as well as females [1]. CHD which is also referred to as Myocardial Infarction (MI) claims the highest share that is approximately 80% out of the total deaths due to CVD [1]. Current projections suggest that there are more than 45 million estimated patients of coronary artery and associated diseases only in India. An alarming number people who are in the age bracket of 30 – 45 years are falling prey to the disease due to factors like sedentary lifestyles (unhealthy eating habits, intake of high sugar and fat containing products), higher rate of smoking and hypertension [2].

### I. Coronary Heart Disease

Coronary (or ischemic) heart or (artery) disease (CHD or CAD) is stated as a physiological condition where the blood vessels that supply oxygen rich blood to the heart muscles, get restricted or blocked, that subsequently leads to the deficiency of oxygen rich blood to the specific heart tissues. Under hypoxic, the heart muscles lose their efficiency to function normally. Coronary arteries are the blood vessels that supply nutrition and oxygen to the heart muscles. With the passage of time, these blood vessels accumulate patches of fat laden with other components, leading to a disorder called atherosclerosis, Fats, lipids, calcium, cholesterol, cellular fragments, etc. circulating in the blood stream get trapped or entangled and get deposited on the inner linings of the coronary arteries and form plaque(s). These plaques continue to grow with time. With the increase in the size of the plaques, the lumen of the affected artery reduces. The plaques are normally hard on the crust, but are soft at the core. Due to the pulsing action of the arteries and the increasing pressure of the rushing blood, these plaques sometimes crack or break apart. This cracking most of the times ruptures the tissues underneath, causing internal bleeding.



**Figure 1:** Sequence of events that lead to myocardial infarction

Whenever there is a tear or injury in the human body that leads to breakage of blood vessels or capillaries, blood starts to ooze out. To reduce the loss of blood the haemostatic system attempts to stop the bleeding. This is achieved by the formation of clot(s) at the bleeding site (Fig 1). There exists a fair amount of understanding about the physiology and the mechanism of the formation of blood clot [3]. The clot formed is a meshwork of protein fibers mainly involving fibrinogen, other blood components such as platelets, broken RBCs, circulating lipids and proteins. When there is injury inside the lacuna of coronary arteries, clotting mechanism attempts to stop the internal bleed inside the blood vessel. As the clot is formed, the room for blood circulation reduces. In severe cases, the flow of blood reduces from 90-99 % due to the blockage created by the clot. The clot formed, blocks the flow of blood, hampering normal circulation, leading to serious consequences including death. If this happens in brain, it's called stroke, if it occurs in coronary arteries, it's known as heart attack. This phenomenon is very common and is normally observed in the blood vessels supplying blood and oxygen to the heart muscles. If the haemostatic system fails to dissolve the blood clot rapidly and efficiently, the surrounding muscles start to die that leads to acute myocardial infarction, stroke, etc. Dissolving and disintegrating the clot becomes a matter of urgency. A timely action can lead to dissolution of the blood clot and relieve the patient from the symptoms of heart attack and even save his / her life. Treatments require clinical intervention consisting of physical methods like angiography and angioplasty, or the intravenous administration of thrombolytic agents [4, 5].

The human body possesses its own clot dissolving system to get rid of the blockages formed due to clots. The dissolution of blood clot is called thrombolysis or fibrinolysis. The principal component responsible for fibrinolysis is an enzyme called plasmin. Plasmin is a serine protease, very similar to trypsin [6, 7]. Plasmin is present in micro-quantities in circulating blood and it's an active form of a pro-enzyme called plasminogen. The fibrinolytically inactive plasminogen on the other hand is present in sufficiently large quantities in the circulatory system. Conversion of inactive plasminogen into plasmin requires a very specific proteolytic cleavage, interceded by a variety of plasminogen activators [8, 9]. Apart from a number of plasminogen activators present in the nature, few have been discovered. These are tissue-type plasminogen activators (t-PA) and urokinase type plasminogen activator (u-PA). t-PA and u-PA are under strict modulation by inhibitors of plasmin activators, e.g. plasminogen activator inhibitor-1 (PAI-1), it's a quick-acting blocker of t-PA and u-PA) and plasmin (e.g.  $\alpha_2$ -antiplasmin and  $\beta_2$ -macroglobulin) [5]. Chemical or biological agents that are capable of dissolving the blood clot are called as thrombolytic agents. These drugs broadly have the property of activating plasminogen into plasmin, the enzyme responsible for dissolving the blood clots. Subsequently plasmin acts on blood clots and results into their dissolution. The blood clotting mechanism is briefed in figure 2.

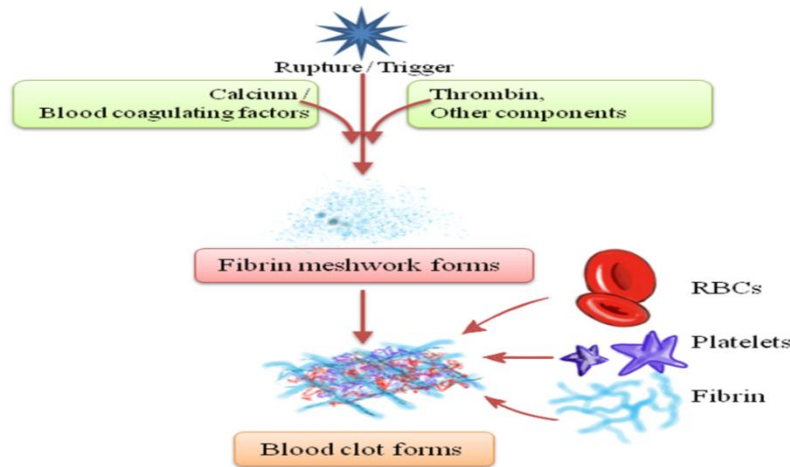
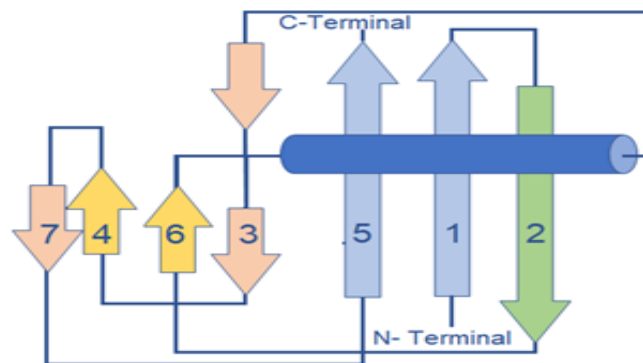


Figure 2: Formation of blood clot

Figure 3: Ribbon diagram for Staphylokinase



### The Existing Therapies

All of the existing therapies for heart attack intend to target the clearance of blockage present in the coronary artery, enabling reperfusion in a timely and effective manner. The dissolution of the blood clot restores the fresh flow of oxygenated blood. This rejuvenates the muscle tissues and reduces the risk of further complications. However, the benefits of reperfusion are highly dependent on multiple factors; e.g. the severity of the blockage, the time taken to report and the time of administration, the physiological state of the blood clot, etc. Currently, there are only few choices to be selected from. The clot is removed by surgical means, primarily by coronary angioplasty and subsequent clearance of the blood clot by chemical or enzymatic means using thrombolytic agents. Angioplasty dilates the coronary artery concerned, with the help of inflatable catheter and subsequently placing a stent together with a platelet anti-aggregation treatment (aspirin, clopidogrel, etc.). As per the chemical or enzymatic treatments, in the case of chemical or enzymatic treatment, the patient is injected with thrombolytic agents that bring the dissolution of the blood clot. The choice is in between chemical or biological agents. The potential biological thrombolytic agents used are urokinase, t-PA, and their derivatives, (e.g. tenecteplase, reteplase, pro-urokinase, etc) and Streptokinase.

### II. Drawbacks Of Existing Therapies

The drugs that are developed via molecular biology techniques and genetic engineering are subjected to modifications from time to time, as better alternatives / derivatives tend to evolve. Till date there have been many derivatives that are under development and clinical trials. Still no clear winner has emerged. Almost all thrombolytic agents are associated with risks and drawbacks. Apart from being expensive, these agents are immunogenic and can be administered only once or a maximum of twice. Therapies offer a very narrow window of operation and induce bleeding complications by their inherent mode of action. Often systemic plasmin generation leads to severe bleeding complications that may lead to intra-cranial bleeding and hemorrhage. Succumbing to these inadequacies, there is a dire need for a better thrombolytic agent with minimum or no haemorrhagic complications. Many thrombolytic agents have been developed and are available with promising

therapeutic potential but none of them can overcome the drawbacks presented by their earlier derivatives. The comparison of various thrombolytic agents is mentioned in Table 1.

**Table 1: Comparison of various thrombolytic agents**

Generation of thrombolytic	Non-fibrin Specific	Fibrin Specific	Approved for clinical use	Plasma Half life	Systemic fibrinogen depletion
First	Streptokinase	-	Yes	18 min	Marked
	Urokinase	-	Yes	15 min	-
Second		Recombinant Tissue Plasminogen activator	Yes	-	Mild
Third	-	Alteplase	No	4 – 8 min	Mild
	-	Staphylokinase	No	-	-
	-	Chimeric thrombolytics	No	-	-
	-	Retepase	Yes	11 – 14 min	Moderate
	-	Tenecteplase	No	20 min	

### Staphylokinase - A potent thrombolytic agent

Staphylokinase (SAK) is approximately a 15 kD molecule, produced by some strains of Staphylococcus, namely *S. aureus*. SAK is supposed to have a huge potential to be used as a thrombolytic agent. The gene expressing Staphylokinase, from Staphylococcus has been cloned and easily expressed in Escherichia coli, producing SAK in gram quantities [9]. This will noticeably bring down the cost of its production. Hence, the formulation is expected to be cheaper and available to a larger spectrum of people. Tissue Plasminogen Activator (t-PA), Streptokinase, urokinase and their derivatives are some of the agents developed or are still under development to be used as drug. SAK is also one such agent having excellent thrombolytic potential [10]. Many trials have been conducted for comparing the clinical efficacy of SAK with other thrombolytic agents, revealing a very clear picture of the performance of these agents. SAK scores much better than the other agents derived from biological sources [11, 12, and 13]. None of these agents possess thrombolytic activity on their own, but these recruit the circulating plasminogen and convert them to active plasmin. Unlike t-PA and u-PA, which are proteases, SAK possess no inherent fibrinolytic activity [7]. Staphylokinase acquires its fibrinolytic activation property by forming a complex with circulating plasmin. This results into 1:1, high-affinity stoichiometric complex, which has a very high specificity and converts other plasminogen molecules to plasmin proteolytically. Thus, the plasminogen activation mechanism of Staphylokinase is entirely different from the proteolytic action and activation by t-PA and u-PA.

Staphylokinase (SAK) is a small protein molecule, made up of 136 amino acids. SAK belongs to the family of staphylococcal proteins. *S. aureus* synthesizes SAK primarily in the late exponential phase of its growth, where it needs to invade the host tissues by dissolving blood clots formed at the site of injury [14]. Mutations in SAK have led to the classification of SAK into 4 different variants. The differences arise due to change in the amino acid sequence at mainly 4 different positions [4, 15, 16, 17, and 18]. The term Staphylokinase was coined when it was found that SAK possessed fibrinolytic activity, like Streptokinase [19]. During the early years (1964) of research on SAK, it was found that it was poorly thrombolytic and induced severe bleeding complications in canine species. SAK scavenged all the fibrinogen from the system [20, 21]. Owing to these complications, the interest in SAK was diminished and virtually abandoned. This is a classic example, where studies on one species cannot be extrapolated to another species. However, these studies were misleading in the retrospective because canine fibrinolytic system is unusually sensitive to systemic activation with Staphylokinase [22].

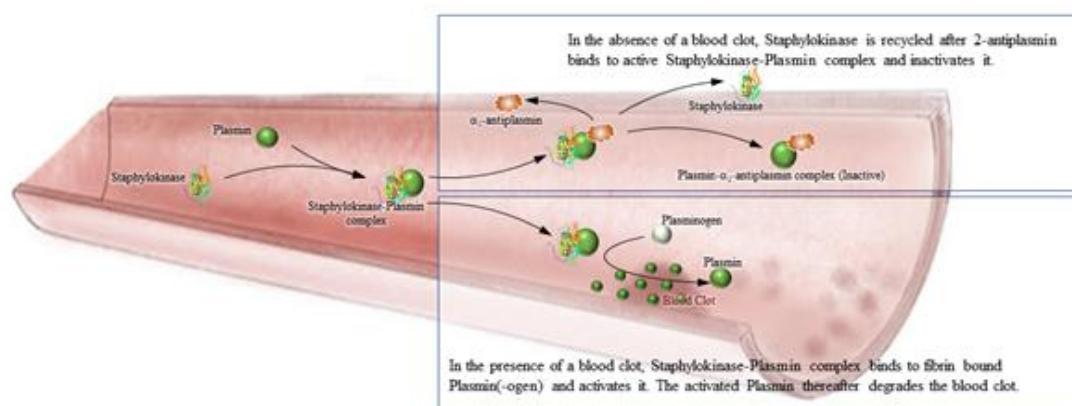
### III. Structure And Properties Of Staphylokinase

The parent gene for SAK transcribes a protein consisting of 163 amino acids. After being transcribed, the 163-amino acid stretch is matured subsequently and processed into a 136-amino acid protein. Essentially Staphylokinase contains an alpha helix and a beta sheet plated on to each other. SAK is a single domain protein [23]; made up of single chain polypeptide and has no disulphide bridges. Based on classification according to SCOP, SAK resembles like an extended dumb-bell shaped molecule. It structurally resembles the members of the  $\square$ -grasp family of protein [24]. The beta sheet is made of five mixed strands packed over an alpha helix of 12 residues. Strands 1 and 5 are parallel strands while 3, 4, 5 and anti-parallel and position adjacently [25]. From the 16<sup>th</sup> residue, Staphylokinase folds into a compact, relatively flat and elongated structure resembling an ellipsoid (Figure 3). The principal axis measures 54 Å; while the other two axes measure 42 Å and 30 Å [23]. Similar to the other members of the b-grasp family, the core of SAK is made up of highly hydrophobic amino acids. The functionally important moieties of SAK real multi-faceted interactions; namely, complex formation with plasminogen is catered by amino acids 46 and 50, induction of active site exposure in plasminogen is carried out by amino acids 65 and 69. These regions face the same side of the molecule. Therefore, it can be concluded that the binding and induction of active site in plasminogen happen in a sequence without any proteolytic cleavage or restructuring of the SAK-Plasminogen complex. The purported N-terminal that's considered important for the activity of the protein is also found on the same side of the molecule [26, 27].

### IV. Mechanism Of Action

- Plasminogen activation, mediated by Staphylokinase is a complicated process and differs from streptokinase-mediated plasminogen activation. These points can be summarized as:
- The Staphylokinase–Plasminogen complex is inactive enzymatically and needs trace amounts of plasmin to get activated.
- Staphylokinase–plasmin complex once activated converts plasminogen to plasmin, but on the other hand it is efficiently inactivated by  $\alpha_2$ -antiplasmin.
- Association of  $\alpha_2$ -antiplasmin to the Staphylokinase-Plasmin complex dissociates SAK from the Staphylokinase-Plasmin complex without altering its catalytic potential and renders it free to bind to other plasmin (-ogen) molecules again [28, 29, 30].
- SAK primarily activates fibrin-bound plasminogen [31] (Figure 4).

Figure 4: Staphylokinase and its action in presence or absence of blood clot



Plasmin is formed in the human body by the spontaneous conversion of plasminogen into plasmin. The process is slow and weak; this leads to traces of plasmin to be always present in the blood. As Staphylokinase is injected into the circulatory system, it encounters the circulating plasmin, that is already generated and circulating in the blood flow. SAK also forms a weak complex with plasminogen, but the affinity of SAK for plasminogen is 160 folds less than that of plasmin [32]. Therefore, SAK doesn't activate circulating plasminogen extensively. In the primary SAK – plasmin complex, SAK is cleaved at the Lys10 - Lys11 peptide bond. This complex then transforms SAK–plasminogen complex to SAK–plasmin complex, which in turn translates circulating plasminogen in to plasmin. The well-known kringle domains of plasminogen do not participate in the interaction with SAK, but with plasminogen. There have been studies that demonstrate Arg719 in plasminogen and Met26 in SAK are crucial for binding with plasmin(-ogen) [18, 26]. The N-terminal of Staphylokinase molecule is essential for the restructuring of the active-site of plasmin molecule [33]. SAK being an asymmetric molecule with disproportionate distribution of its hydrophobic and hydrophilic residues has been considered imperative for its activation [34]. Substitution of Lys11, Asp13, and Asp14 at the amino terminal of SAK with Ala, resulted

in a transmuted SAK. The mutated SAK retained affinity towards plasminogen but lost the potential to convert soluble plasminogen into plasmin. Similarly, substitution experiments with Glu46 and Lys50 as well as Glu65 and Asp69 resulted into SAK that had much lower affinity for plasmin(-ogen) that consequently impaired the conversion of plasminogen to plasmin [27]. When there is no fibrin present, the creation of the SAK – plasmin complex in the plasma milieu and other physiological fluids was competently inhibited by  $\alpha_2$ -antiplasmin, preventing subsequent plasminogen activation. However, inhibition of SAK-Plasmin complex formation does not occur, if the lysine-binding moieties of plasmin in the SAK-plasmin complex are unavailable due to binding to fibrin or fibrinogen fragments [30]. SAK also binds much more efficiently to fibrin-bound plasminogen than to soluble plasminogen [32]. The fact that SAK principally activates plasminogen adhered to fibrin without resulting in systemic plasminogen activation and not inducing systemic bleeding has up stretched the interest to exploit SAK as an effective thrombolytic agent in solubilizing and degrading the fibrin associated with blood clots [31, 35, 36].

### **V. Advantages Of Staphylokinase**

Staphylokinase has some distinct advantages over the other existing thrombolytic agents. To list down a few:

Traces of plasmin, even up to 3 ppm, in circulation system can initiate the activation of SAK and result in the formation of SAK-Plasminogen complex [21, 36]. Staphylokinase is not activated in the plasma milieu if there is no plasmin. SAK affinity for free- or fibrin bound plasminogen is very less [23]. Fibrin as such has no effect on the activation or activity of Staphylokinase [37]. In circulation, in the absence of clot,  $\alpha_2$  – antiplasmin removes free plasmin and impedes the formation of SAK – Plasmin(-ogen) complex. Complexes formed, if any are rapidly neutralized by  $\alpha_2$  – antiplasmin, releasing active Staphylokinase molecule. Hence the SAK molecule is recycled, and hence the required dosage may reduce. In the plasma milieu, in the presence of fibrin clot, traces of plasmin are present (due to the physiological plasminogen activation). These plasmin molecules are bound to the clot via lysine binding site and therefore protected against rapid inhibition by  $\alpha_2$  – antiplasmin. Hence, Staphylokinase is processed locally around the clot and its activity remains concentrated around the clot. Any SAK – plasmin complex if released from the clot into circulation is rapidly inhibited. This makes Staphylokinase clot specific and refrains it from systemic generation of plasmin (Figure 3).

Staphylokinase when compared to streptokinase is found to be more active towards platelet poor (unretracted) and platelet rich (retracted) clots. Streptokinase is only active against platelet poor clots [28]. This property is of significant clinical implication as the high platelet content of a coronary thrombus, along with retraction and ageing, are believed to significantly limit the thrombolytic efficiency of conventional non-fibrin selective agents.

### **VI. Production Of Staphylokinase**

Considering the collective incidences of coronary heart diseases and the therapeutic potential and massive applicability of Staphylokinase, attempts have been made in the recent past to produce Staphylokinase by other alternative routes, with the principle objective to get high production levels and higher specific SAK expression per unit volume of fermentation medium. Several systems have been designed and devised for production of Staphylokinase. Staphylokinase is mainly produced by *Staphylococcus* sp., which is the normal flora of the skin, intestine, etc. *Staphylococcus* is pathogenic, slow growing and requires stringent precautions while handling this organism, for fermentation studies. Handling hazardous organism requires special equipment(s) and containment facilities. Therefore, attempts have been made to clone the Staphylokinase gene in other non-hazardous microbes, so that the culture and production can be made easy. At laboratory scale, native organism produces only 300  $\mu\text{g}$  of Staphylokinase per liter of culture broth and the purification yield goes down to  $4\mu\text{gL}^{-1}$ , which is not feasible or economical for large-scale production [38]. In order to increase the production, SAK gene was cloned in *E. coli* to make the economical and profitable production of Staphylokinase. The overproduction of recombinant Staphylokinase has already been reported by few groups with production up to 913 mg/L [38] and 3600 mg/L [9]. Production of recombinant Staphylokinase is always advantageous over the natural strain in terms of product titer and Staphylokinase productivity.

### **VII. Conclusion**

As the lifestyle diseases are on the rise, the number of people suffering from these is increasing exponentially. With WHO confirming with year on year survey, CVDs are the leading cause of death and disability. The number is destined to increase even faster. Myocardial infarction is one of the major culprits for the CVD associated deaths. This situation leads to huge demand for an effective thrombolytic agent. There are very small number of peptides and proteins derived from microbial or animal sources that appear capable in the solubilizing blood clots. However, none of the agents researched have been reported free from potential side effects or drawbacks. SAK is one potent anti-thrombolytic agent which has many benefits, compared to

drawbacks. The small size of Staphylokinase makes it less immunogenic than others, the small size also allows SAK to move unrestricted inside the un-retracted as well as retracted blood clots. This effectively increases the surface area for the action of SAK and hence the clot may dissolve much faster; this m. SAK does not elicit an elevated immune response from the host machinery, as in the case of Streptokinase and other proteins microbial proteins. It doesn't induce systemic plasmin generation therefore, doesn't lead to internal bleeding. Staphylokinase thus proves its self to be one of the potential molecules that if pursued and researched can be one of the best thrombolytic molecules to be administered for myocardial infarction.

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#### **Conflict of Interest**

The authors declare there was no conflict of interest

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