Removing plasmin from the equation: something to chew on…

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Removing plasmin from the equation – something to chew on….

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Thrombolytics or fibrinolytics are a group of pharmacological agents used to target and dissolve occlusive intravascular thrombi. Thrombi form a haemostatic plug at the site of injury to arrest bleeding and are essential for wound healing[1]. However, intravascular thrombi that aberrantly form in pathophysiological settings block blood vessels lead to disturbed blood flow, thereby promoting thromboembolic events. Degradation of a thrombus occurs when the circulating zymogen, plasminogen, is cleaved to an active serine protease, plasmin[2,3]. This process, termed fibrinolysis, is dependent on the presence of plasminogen activators; namely tissue plasminogen activator or urokinase (tPA or uPA, respectively) [2,4]. The differences in the mechanism of action of tPA and uPA are also important, tPA requires fibrin as a co-factor to form a tertiary complex with plasmin[5,6], however, uPA does not and can promote plasmin generation in solution or on the cell surface[7-9]. tPA is also more susceptible to plasminogen activator inhibitor 1 (PAI-1) inhibition, as demonstrated by their second order rate constants, which differ by an order of magnitude; 12.6×10^7 *vs.* 4.8×10^6 $M^{-1}s^{-1}$ for tPA and uPA, respectively[10].

Current licensed thrombolytic therapies include direct plasminogen activators that convert plasminogen into plasmin. These encompass urokinase and recombinant forms of tPA; Alteplase, Reteplase and Tenecteplase. Alteplase, is a first generation thrombolytic that is identical to the native plasminogen activator tPA. Tenecteplase is a bioengineered variant of tPA with enhanced fibrin specificity, reduced affinity for the endogenous inhibitor plasminogen activator inhibitor 1 (PAI-1) and a prolonged half-life *in vivo*[11]. Tenecteplase has been shown to be efficient in treatment of acute myocardial infarction. The first licensed thrombolytic, streptokinase, is an indirect plasminogen activator that functions by forming a 1:1 high affinity complex with circulating plasminogen giving rise to a conformation change in the protein which provokes proteolytic activation of other plasminogen molecules[12]. These indirect plasminogen activators, streptokinase and staphylokinase, are of bacterial origin and have no direct enzymatic activity[13]. Staphylokinase is not currently licensed but has several advantages over streptokinase, as it shows a degree of fibrin specificity thereby limiting systemic degradation of fibrinogen. The resulting complex is also resistant to inhibition by the principal inhibitor of plasmin, α_2-antiplasmin (α_2AP)[14].

Current thrombolytic agents are compromised by an unfavourable safety profile, arising from off target effects leading to haemorrhagic complications and tissue

damage[15-17]. These undesirable qualities are attributed to systemic activation of plasminogen promoting fibrinogenolysis. Unrestrained proteolytic activity of plasmin, can result in degradation of proteins and molecules that function in tissue repair and wound healing, including fibronectin and vascular endothelial growth factor (VEGF) [18-21]. Currently, Alteplase is the only licensed therapy for acute ischaemic stroke and must be administered within a 3 – 4.5 h therapeutic window from onset of symptoms to limit neurological complications[22-24]. This is an extremely narrow time frame to permit hospital admission of the patients and associated computed tomography scan, to exclude haemorrhagic stroke, prior drug administration. There have been multiple clinical trials investigating alternative thrombolytic therapies as a treatment for acute ischaemic stroke, but these have been terminated early due to unacceptable rates of intracranial haemorrhage (reviewed by[25]). The lack of thrombolytic drugs available to treat stroke, and other thromboembolic complications, and current safety concerns highlight the necessity for a new class of thrombolytics with an enhanced safety profile and increased window of administration. A prototypical thrombolytic drug would demonstrate fibrin specificity (to localise the mechanism of action to the clot), resistance to inhibition, low incidence of haemorrhage, reasonable cost, straightforward administration and no antigenicity.

An interesting article published in *Circulation Research* in February 2021, reports on a novel thrombolytic agent that functions independently of plasmin[26]. This family of proteins, termed high-temperature requirement A (HtrA) proteins, are vital for prokaryotic survival and are highly conserved in nature[27-29]. Intriguingly, HtrA are serine protease enzymes akin to the enzymes of the coagulation and fibrinolytic pathways. HtrA proteins are heat shock proteins that function in the degradation of misfolded proteins within bacteria in response to cellular stress conditions[30-32]. HtrA proteins are evolutionarily conserved with divergence into four genes in mammals[28]. Of note, HtrA1 has been shown to degrade misfolded tau protein aggregates in Alzheimers disease[33]. Thrombi are essentially a mass of highly damaged and aggregated proteins, with the scaffold protein, fibrin, reported as a misfolded protein[34].

Hassan *et al*[26] hypothesised that the mammalian derived heat-shock, HtrA proteins, may function in degradation of thrombi. They applied a combination of *in vitro* and *in vivo* experiments to analyse the mechanism of action of HtrA proteins and compare the fibrinolytic profile to conventional thrombolytics, namely plasmin, streptokinase,

urokinase or tPA. HtrA1 and HtrA2/Omi were able to degrade fibrin in a plasmin-independent manner. Cleavage occurred at distinct sites in the α, β and γ chain of fibrin whilst no cleavage of native fibrinogen or fibronectin was observed. In a mouse tail bleeding model HtrA proteins dissolved intravascular thrombi without targeting the wound healing process. Analysis of clotting times showed a significant prolongation in the conventional arms, but not in the HtrA treatment group. *In vivo* models of thrombosis, including pulmonary emboli and $FeCl_3$-induced thrombosis in the carotid artery, revealed that HtrA proteins were able to promote dissolution of thrombi and increase survival rates. These data indicate that HtrA proteins have the potential to drive thrombolysis while preserving the innate wound healing response. It was unclear from the report whether fibrin degradation products were cleared from the circulation in the same manner as those produced with conventional plasmin cleavage, or what the half-life of these of these proteases are *in vivo*.

A notable finding of the study is that HtrA proteins were found to effectively dissolve platelet aggregates[26], a characteristic that is not associated with conventional thrombolytic agents. The authors indicate that HtrA proteins did not target circulating platelets, but the evidence and experimental set up of these experiments was not clear. They did show that HtrA proteins degraded the integrin αIIbβ3 in aggregated platelet lysates. The integrin αIIbβ3 is the most abundant glycoprotein receptor on the platelet membrane[35,36] and is essential for platelet activation and aggregation, by permitting fibrinogen to act as a molecular bridge between platelets[37,38]. It is unclear from the observations whether this attribute of HtrA proteins could negatively impact on their usefulness, as this action may interfere with primary haemostasis thereby promoting haemorrhagic complications. Further work is required to fully unpick the mechanisms underpinning this group of novel plasmin independent thrombolytics and specifically to define the impact on platelets and clot initiation.

There is a growing field of research to identify novel, safe and effective thrombolytics (**Figure 1**). Most recently, Huang et al have developed nanovesicles which mimic fibrinogen to allow targeted delivery of tPA to the site of injury[39]. The nanovesicle is coated with polyethylene glycol (PEG) conjugated to a cyclic RGD peptide which fuses with the platelet membrane upon binding to the liposomes of activated platelets[39]. tPA has a short circulation time, 2-6 min, as it is rapidly inactivated by PAI-1 or removed by the liver[40,41]. The development of nanovesicles to encapsulate and protect tPA in

the circulation and target the site of the thrombi overcomes the failings of native and recombinant tPA. A recombinant microplasmin molecule (HtPlg) targeted to the platelet surface via an activation-specific αIIbβ3 single-chain antibody (SCE5) has also been described[42]. Microplasmin is a truncated form of plasmin that lacks the kringle domains of full length plasminogen[43] and is less susceptible to inhibition by the principal inhibitor of the serine protease, α_2AP[44]. The resulting microplasmin fusion protein, SCE5-HtPlg, targets αIIbβ3 on the platelet surface and is activated locally by thrombin to release the active microplasmin[42]. Alternative thrombolytic delivery systems include microwheels (μwheels), whichuse superparamagnetic colloidal beads and magnetic fields to roll along surfaces at high velocity. These μwheels can be loaded with therapeutic agents that are delivered to the site of thrombosis[45]. The μwheels platform is being developed to co-deliver tPA and plasminogen to overcome several limitations of current thrombolytic therapies, including plasminogen consumption[45,46].

Targeting of inhibitors of fibrinolysis has been considered as an alternative to thrombolytics to promote fibrin degradation by enhancing endogenous plasmin generation or activity. Small molecules, peptides, monoclonal antibodies and antibody fragments have been used to modulate PAI-1 activity by interfering at different stages of the PAI-1/plasminogen activator interaction[47-51]. Drugs targeting PAI-1 in the experimental phase have produced promising results[52-54]. A potent neutralising diabody to PAI-1 and activated thrombin activatable fibrinolysis inhibitor (TAFIa) rapidly enhanced clot breakdown[53]. Simultaneous inhibition of PAI-1 and TAFIa may improve current thrombolytic therapy; for example, co-administration with tPA may permit a lower dose and thus enhance its safety profile[53,55]. The therapeutic potential of α_2AP inactivation, the physiological inhibitor of plasmin, has also been explored. Potent and specific inhibitors to human α_2AP were found to rapidly degrade thrombi *in vitro*[56-60] and several *in vitro* mouse models have found that inhibition of α_2AP reduces ischaemic stroke injury[61-63]. These promising lines of attack provide alternatives to conventional thrombolytic treatment that unleash the proteolytic activity of plasmin systemically and are more likely to enhance the local endogenous activity of plasmin at the site of thrombosis.

The interesting observations in the manuscript by Hassan *et al*[26] provide intellectual nourishment regarding alternative approaches to thrombolysis. If we remove plasmin

from the equation can we safely dissolve thrombi in the body while preserving normal haemostasis and wound healing? Certainly, this would be a revolution in thrombolytics that could boost favourable safety profiles. Mutations in the serine protease domain of HtrA1 have been reported in inherited cerebral small vessel disease, providing evidence of a physiological link between this family of serine proteases and cardiovascular diseases[64-66]. It remains to be seen whether HtrA proteins would have substantial benefit over other novel approaches to deliver thrombolytic drugs to the site of thrombosis that promote thrombolysis via a traditional plasmin-mediated pathway or indeed over targeting the fibrinolytic arm to promote endogenous lysis. Nonetheless, it is evident we are in an exciting era of discoveries and novel technologies which will revolutionise thrombolytic therapy.

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Conflicts of Interests

G.B.M and N.J.M have no relevant conflict of interest to declare.

Author Contributions

G.B.M researched, wrote and edited the manuscript. N.J.M conceived the idea and edited the manuscript.

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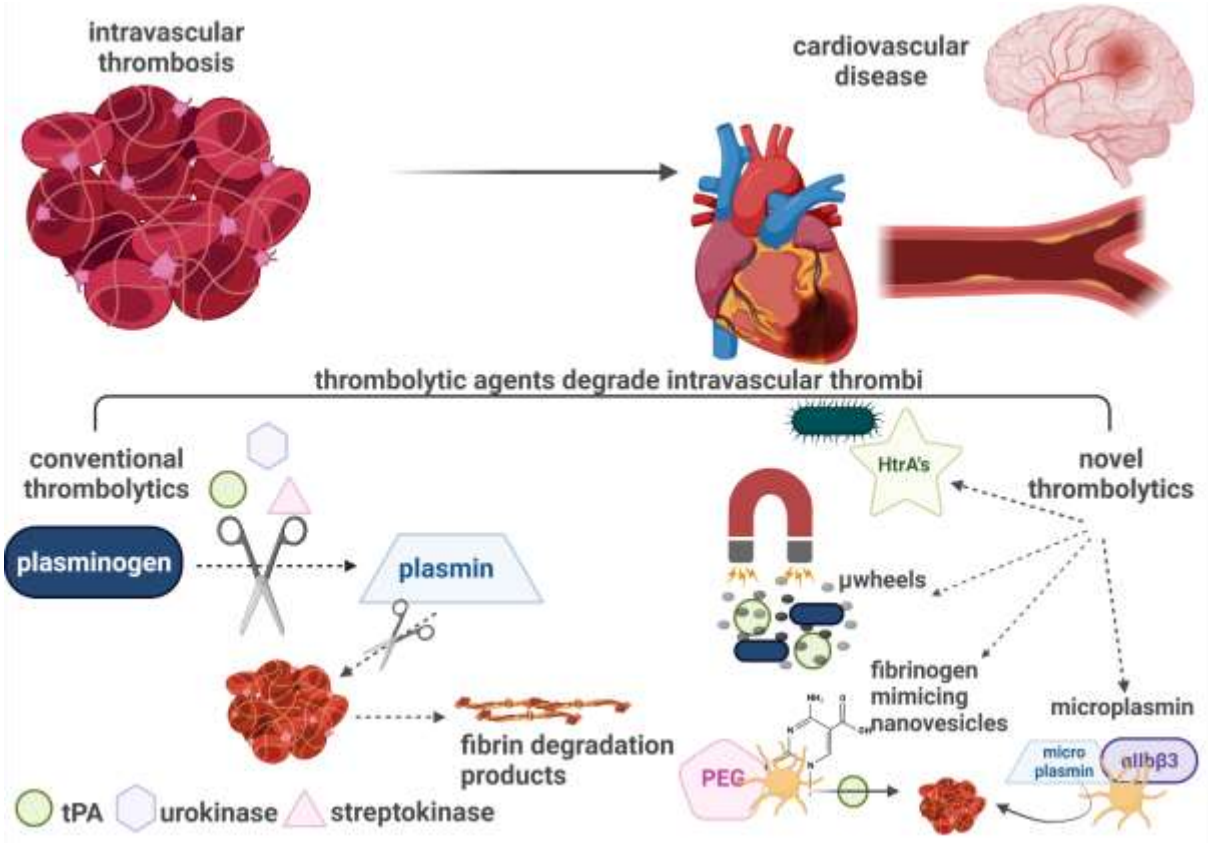

Figure 1 Novel thrombolytic agents under development

Intravascular thrombi that occlude blood vessels disturb blood flow and lead to development of cardiovascular disease, such as ischaemic stroke, myocardial infarction, venous and arterial thrombosis. Thrombolytics are a class of drugs utilised to degrade intravascular thrombi. Conventional thrombolytics, such as tissue plasminogen activator inhibitor (tPA), urokinase and streptokinase, activate plasminogen into an active serine protease, named plasmin. Plasmin is the principal enzyme that degrades fibrin, the main constituent of a thrombus, into fibrin degradation products. Conventional therapeutic use of thrombolytics is limited due to their unfavourable safety profile leading to bleeding complications and additional off target effects. Novel thrombolytics that are highly effective and safe are under development in several laboratories. A novel delivery mechanism is the use of microwheels (μwheels), which use superparamagnetic colloidal beads and magnetic fields to roll along surfaces at high velocity. The μwheels co-deliver tPA and plasminogen to the site of the thrombus therefore targeting delivery and promoting dissolution. Nanovesicles composed of a polyethylene glycol (PEG) and cyclic RGD peptide that mimic fibrinogen binding to the platelet membrane are being developed to deliver tPA

to the thrombus at the site of injury. The utilisation of a microplasmin molecule that is fused to an αIIbβ3 antibody target the activated platelet membrane, promoting localised clot dissolution and interaction with the membrane prevents premature degradation by α_2-antiplasmin. High-temperature requirement A (HtrA) proteins play an important role in the degradation of misfolded proteins in bacteria. Mammalian derived HtrA protein function have been investigated as an alternative thrombolytic therapy on the premise that fibrin is a misfolded protein. HtrA act independently of plasmin, lowering the risk of adverse bleeding function and have potential in directed therapy against aberrant blood clots. Diagram created using Biorender.