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Vascular targeting of anti-thrombotic agents

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INTRODUCTION

Thrombosis, i.e., formation of pathological intravascular blood clots is the most common cause for obstructive cardiovascular diseases leading to ischemic damage to the involved blood vessels and tissue ischemia (1). Pathologically altered vasculature (e.g., in inflammation sites) is predisposed for thrombosis, in part due to suppression of natural anti-thrombotic mechanisms in endothelium lining vascular lumen (2). Activation of the coagulation cascade generates thrombin that cleaves fibrinogen, producing a fibrin meshwork that along with the activation of platelets forms large intravascular aggregates (2). In the venous vasculature, clots are red and consist predominantly of fibrin and entrapped red blood cells (RBC), whereas in the arterial vasculature clots formed at high shear stress are mostly populated by platelets (2).

Approaches to pharmacologically manage thrombosis include prevention and therapy (Figure 1). Prevention is attained by prophylactic use of anticoagulants and platelet inhibitors (3). Anticoagulants with a delayed onset and relatively prolonged effect are used for long-term prevention (e.g., Warfarin), whereas thrombin inhibitors heparin and hirudin act within minutes and can be used for an immediate short-term thromboprophylaxis (4). A second approach is emergency therapy of thrombosis which employs intravascular injection of plasminogen activators (PA) (5). These middle-size proteases (50–60kD) include tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) that generate plasmin, which cleave fibrin clots and help to restore perfusion. Table 1 introduces current anti-thrombotic agents.

Alas, both approaches provide rather limited efficacy of action and are liable in serious side effects, first of all, bleeding. Agents that act expeditiously (e.g., fibrinolytics) are rapidly cleared from the bloodstream. Several strategies to prolong the half-life in the circulation have been devised including structural modification and mutation of a protein drug to remove clearance recognition sites and replace labile amino acids, conjugation with PEG and application of drug delivery systems such as liposomes and biodegradable particles (6–9). However, despite these efforts, delivery of anti-thrombotic drugs to the site of their preferential effect, i.e., vascular sites predisposed to thrombosis or nascent intravascular clots, remain grossly suboptimal. A major fraction of the injected drug is an expensive and dangerous waste. In particular, impermeability of occlusive clots restricts therapeutic fibrinolysis by PA. Within minutes after infusion, mega-doses of fibrinolytics (e.g., 100mg of t-PA) are needed to overcome its inefficiency and achieve fibrinolysis locally, excess drug diffuses into pre-existing hemostatic mural clots predisposing to bleeding and into tissues such as the brain (Figure 1).

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In theory, targeted delivery of anti-thrombotic agents, either to pathological vessels or into nascent clots may help solve the problem (10). Cardiovascular disease and thrombosis are typically located to discrete vascular regions, affording opportunity for targeted anti-thrombotic pharmacotherapy. This review will highlight the studies on affinity targeting anti-thrombotic therapeutics to fibrin, platelets, red blood cells (RBC) and endothelium.

Vascular targeted delivery of anti-thrombotic agents: drug targeting to fibrin and platelets

Since the fibrin molecule exposes epitope determinants absent on fibrinogen, it has been viewed as a molecular target for delivery of thrombolytic agents selectively to the thrombi (5,11). Diverse anti-thrombotic agents including hirudin and plasminogen activators have been conjugated with fibrin-specific antibodies and their fragments. For example, in rabbit and baboon models of jugular vein thrombolysis, an anti-fibrin/tPA conjugate proved to be more potent vs. tPA (12–13). Conjugation of a single chain urokinase type plasminogen activator (scuPA) to anti-fibrin has also resulted in marked increase in potency vs scuPA (14–15). Anti-fibrin coated nanoparticles conjugated with streptokinase (SK) have also been shown to be more effective in clot lysis vs. free SK *in vitro* (16).

However, a conundrum in targeting fibrinolytics to fibrin (or other specific clot components) is that the target does not exist before the thrombosis, whereas these drugs do not circulate for a prolonged time. This, and side effects of systemically active fibrinolytics preclude prophylactic use. On the other hand, after thrombosis majority of the formed clot mass becomes inaccessible to drugs. In fact, somewhat paradoxically, the higher fibrin affinity drugs have, the more impeded their penetration into the clot, due to retention on the upstream surface layer that works like an affinity column (17).

Platelets have also been used as a target for thromboprophylaxis and thrombolytic therapy. Potential limitations of this strategy include a relatively short life-time of platelets, especially carrying targeted drugs. However, this approach seems especially attractive for interventions in arterial clots. For this purpose, investigators used antibodies and antibody fragments to platelet membrane glycoproteins, such as fibrinogen receptor GPIIb/IIIa (18–19). The conformational changes induced in the GPIIb/IIIa complex by thrombin or collagen markedly enhance fibrinogen affinity (18–19). Ligands that recognize activated GPIIb/IIIa can be used for targeting drugs to platelet-rich clots (with the permeability caveat similar to that of fibrin targeting), whereas ligands binding to either resting or activated targets can be used for loading drugs on platelets prior to thrombosis. In addition, blocking GPIIb/IIIa inhibits platelets, providing the basis for anti-thrombotic effect of Reo-Pro and other anti-platelet agents. For example, urokinase conjugated with GPIIb/IIIa antibody has been shown to bind platelets and platelet-rich thrombi and dissolve arterial thrombi (20). It is likely that some activity of the conjugate could be attributed to blocking of the fibrinogen receptor GPIIb/IIa. Antibodies against other platelet glycoproteins such as GPIIIa and GPIIb, have also been used for the targeted delivery of plasminogen activators (21). In general, targeting anti-thrombotic agents to platelets seems an interesting avenue, worth further development and testing.

Targeting to red blood cells (RBC)

RBCs provide a natural carrier vascular drug delivery (22), in particular, for intervention in venous thrombosis (23). Hemodynamic factors propel RBCs towards the blood mainstream, restricting contact with vascular walls and hemostatic mural clots, reducing side effects. RBCs do not penetrate pre-existing hemostatic clots and restrict effect of the drug in blood. RBCs circulate for several weeks, providing a sufficient prophylactic window. Recent animal studies showed that biocompatible coupling to RBC converts plasminogen activators

from problematic therapeutic agents into effective and safe agents for intravascular thromboprophylaxis, thereby shifting the paradigm of the fibrinolysis (23).

In prototype studies, utilizing homologous RBC carrying chemically coupled tPA, prophylactic infusion of RBC/tPA complexes into rats and mice delivered tPA into the interior of intravascular venous and arterial nascent clots, lysing within and during clot extension in settings where even a 10-fold higher dose of soluble tPA was ineffective (23) (Table 2 and Figure 2A). Coupling tPA to RBC prolonged tPA circulation by orders of magnitude without side effects including bleeding, RBC damage or activation of complement (24–25). In addition, RBC glycocalyx protected tPA from plasma inhibitors (26). Both direct coupling of tPA to RBCs and coupling of urokinase to RBCs carrying conjugated urokinase receptor yielded remarkable improvements in the benefit/risk ratio, reducing side effects and enhancing bioavailability (23, 27).

Furthermore, RBC/tPA has provided effective and safe thromboprophylaxis in more challenging settings such as cerebral embolism, thereby preventing deleterious consequences of brain ischemia. In mice, RBC/tPA dissolved subsequently formed occlusive cerebrovascular thrombi, leading to rapid and stable reperfusion, marked alleviation of ischemic brain injury and improved survival, whereas free tPA failed to provide reperfusion and in fact enhanced mortality (28). In a pig model of cerebral ischemia/hypoxia, RBC/tPA alleviated, whereas tPA aggravated vascular abnormalities (29). A similar outcome has been observed in a pig model of cerebral thrombosis: RBC/tPA restored perfusion and alleviated cerebral abnormalities, whereas tPA aggravated pro-inflammatory and vasoconstrictor side effects (30). Of note, RBC/tPA injected in rats shortly prior or after traumatic brain injury caused no brain hemorrhage, in contrast with free tPA (31).

These animal studies utilizing infusion of RBC carrying conjugated tPA revealed superiority of RBC/tPA thromboprophylaxis vs free tPA use. Subsequent recent studies further translated this promising prototype closer to the potential clinical applications, by advancing the methodology for coupling drugs to RBC, avoiding the need of having to couple tPA to isolated RBC's *ex vivo* followed by transfusion. In the prototype study, tPA conjugated with antibody to RBC determinant CR1 showed binding to circulating RBC after IV injection, leading to prolonged circulation and thromboprophylaxis in a mouse model of thrombosis (32).

Next, in order to avoid technical and regulatory challenges associated with chemical conjugation of antibodies, this molecular format has been replaced by single-chain Fv fragments (scFv, comprising variable domains of heavy chain V_H and light chain V_L). A recombinant fusion protein combining a single chain antigen-binding fragment of a monoclonal antibody against mouse glycophorin A was fused with truncated urokinase providing scFv-uPA (33). Urokinase is an attractive drug for vascular targeting. It exists as an inactive single-chain zymogen of 411 amino acid residues (single-chain uPA, scuPA) that is converted by plasmin cleavage into fully active two-chain uPA (tcuPA) (33). The scuPA consists of three domains: the N-terminal domain homologous to the epidermal growth factor, the kringle domain, and the C-terminal catalytic domain. Urokinase binding to a cellular receptor via the "growth factor-like domain" (GFD) activates vascular cells (34), but deletion of the GFD provides a low-molecular-weight form of scuPA (MW 32-kD) that has enzymatic features similar to those of full-length scuPA. When injected in mice, low molecular weight scuPA fused with anti-GPA scFv (scFv-uPA) safely bound to RBC, which markedly prolonged its intravascular circulation and fibrinolytic activity compared with its non-targeted uPA counterpart, and resulted in prevention of thrombotic occlusion caused by vascular injury (33).

The scuPA zymogen (pro-urokinase) can be activated by trace amounts of plasmin over time and formed tcuPA may cause adverse effects, which would limit prophylactic use. Since tcuPA is rapidly inactivated by plasminogen activator inhibitor (PAI-1), the duration and effectiveness of prophylaxis would also be limited. Further, thrombin inactivates uPA by cleaving Arg156-Phe157, negating its effect at sites of active thrombosis. These problems might be solved by deleting Phe157 and Lys158, which yields a plasmin-resistant mutant activated by thrombin (uPA-T) (35). This pro-enzyme will not be activated by plasmin *in vivo* (thus avoiding systemic effects and premature PAI-1 inactivation), while thrombin will activate it locally at sites of nascent thrombosis within seconds of clotting. In order to further minimize premature activation and localize it to the sites of active ongoing thrombosis, uPA portion of the anti-GPA scFv-uPA fusion has been mutated to replace natural plasmin-sensitive activation site by a thrombin-sensitive one (36). This scFv-uPA-T has been shown to bind specifically to RBC's without altering their biocompatibility, while causing thrombin-induced fibrinolysis even 20 hours after intravenous injection (36). These results provide proof-of-principle for the development of a recombinant PA variant that binds to circulating RBC and provides thromboprophylaxis in the settings associated with high risk of acute severe intravascular thrombosis.

Targeting to endothelium

Endothelial cells line the luminal surface of blood vessels and control vascular tone, blood fluidity and extravasation of blood components. In particular, the endothelium plays a central role in thrombosis and represents a key target for pharmacological anti-thrombotic interventions. The main goal of endothelial targeting anti-thrombotic drugs is for prophylactic purpose, to boost anticoagulant or thrombolytic potency in the vascular areas predisposed to thrombosis. Several endothelial determinants have been employed for this function (10). For example, tPA chemically conjugated with anti-ACE retained fibrinolytic and antigen-binding activities and exhibited sustained preferential accumulation in rat pulmonary vasculature (37). Other endothelial determinants have also been explored for this goal; both tPA and uPA chemically conjugated with antibodies against antigens enriched in the pulmonary endothelium accumulated in the pulmonary vasculature (37–38).

In particular, Platelet-Endothelial Adhesion Molecule-1 (PECAM-1, CD31) and Inter-Cellular Adhesion Molecule-1 (ICAM-1, CD54), transmembrane glycoproteins constitutively expressed on endothelial cells represent attractive targets for anchoring anti-thrombotic agents in the vasculature predisposed to thrombosis or embolism (Table 2). The pulmonary vasculature contains ~30% of the endothelial surface in the body and receives the entire cardiac output and, as a result, agents with an endothelial affinity accumulate in the lungs after intravenous (IV) injection. This vascular bed is an important target for treatment of acute lung injury, oxidative stress, thrombosis and inflammation, among other conditions (39). Agents conjugated with anti-ICAM and anti-PECAM infused IV accumulate in the lungs (40–41), whereas local infusion in a conduit artery enriches the binding in the downstream vascular areas (e.g., cardiac, cerebral and mesentery vasculature) (42–43). PECAM and ICAM are involved in mechanisms of cellular recognition, adhesion and trans-endothelial migration of leukocytes. Thus blocking these molecules may inhibit leukocyte trafficking, a bonus in treatment of inflammation and thrombosis. Endothelial cells do not internalize anti-ICAM and anti-PECAM or their monovalent fragments such as scFv (44–45), which provides the optimal strategic position of anti-thrombotic drugs anchored to these molecules in the vascular lumen.

PECAM is stably expressed on the endothelium at the level of a million copies per cell predominantly localized in inter-endothelial borders, whereas endothelial cells in the vasculature express ICAM-1 at a surface density of 2×10^4–2×10^5 surface copies per cell and this level doubles upon pro-inflammatory challenge (46). Diverse agents conjugated to anti-

PECAM and ICAM accumulate and display their functional activity in the endothelium as soon as 10 min after IV injection in mice, rats and pigs (10). After IV injection in rats, pulmonary uptake of anti-ICAM/tPA conjugate is two orders of magnitude higher than that of control IgG/tPA, which resulted in enhanced fibrinolysis of subsequent pulmonary emboli (47).

Chemical conjugation of proteins with antibodies yields multivalent conjugates that may cause endocytosis undesirable in the context of sustained retention of fibrinolytics on the endothelial lumen (44). In contrast, recombinant fusion of uPA with scFv antibody fragments yields monovalent, homogeneous and relatively small drugs (50–70kD, hence low immunogenicity and lack of Fc-fragment mediated side effects). As a proof of principle for prophylactic thrombolysis by endothelium-targeted thrombolytic fusions, using a linker of three Gly_4Ser repeats, an anti-PECAM scFv was fused with low-molecular weight uPA described above in the context of anti-RBC fusions. After IV injection, the protein composed of an anti-PECAM scFv fused with lmw-scuPA (anti-PECAM scFv/uPA) preferentially accumulated in the lungs of wild-type but not PECAM deficient mice, persisted in the lungs for at least 3 hours and remained on the endothelial surface. Compared with non-targeted uPA, scFv/uPA augmented local lysis of pulmonary emboli in a mouse pulmonary thrombotic model (41). Further, scFv/uPA accumulated in the cerebral vasculature after intra-arterial and IV injection, dissolved cerebral clots and improved blood reperfusion without hemorrhagic complications, thereby mitigating post-thrombotic brain edema in a mouse model of cerebral embolism (48).

Replacing the native plasmin activation site in the uPA moiety of scFv/uPA with a thrombin activation site provided thrombin-activated anti-PECAM scFv/uPA-T (49). This construct was also found to contain an intrinsic thrombin-sensitive cleavage site in the anti-PECAM scFv moiety, providing a built-in mechanism for local drug release. The scFv/uPA-T is latent and resists the PA inhibitor PAI-1 until activated by thrombin. After IV injection in mice, scFv/uPA-T did not consume plasma fibrinogen, in contrast with scFv/uPA that has this liability. However, scFv/uPA-T bound to the endothelium and accumulated in the vascularized organs, particularly the lungs. In a mouse model of thrombin-induced pulmonary thrombosis, scFv/uPA-T provided more potent and durable thromboprophylaxis than both plasmin-sensitive scFv/uPA and lmw-scuPA. Further, injection of mice with scFv/uPA-T prior to unilateral lung ischemia/reperfusion attenuated pulmonary fibrin deposition, to a significantly greater extent than plasmin-sensitive scFv/uPA, and restored arterial oxygen tension, while PAI-1 susceptible plasmin-activated scFv/uPA did not improve blood oxygenation (49). Therefore, vascular targeting (attained by fusion with scFv) of a pro-drug locally activated by the key pathological mediator, thrombin, provides the maximal degree of spatiotemporal precision and efficacy of the pharmacotherapy of occlusive vascular disease.

SUMMARY

Thrombolytic/fibrinolytic agents used for prevention and therapy of occlusive cardiovascular diseases including acute myocardial infarction, pulmonary embolism and thrombosis have limited efficacy and serious side effects. Conjugating these agents, for example thrombolytic plasminogen activators, with affinity carriers that deliver cargoes to blood elements or to the lumen of the predisposed vasculature may improve thromboprophylaxis of nascent pathological clots. In particular, RBC and endothelial cell targeting which has been shown to result in a reduced risk of hemorrhage and toxicity associated with systemic drug administration. Using a modular recombinant format of scFv fragments directed to specific blood cell or endothelial determinants provides a modular approach to achieve this goal. Mutating plasminogen activators such as urokinase in a way

that renders them activated selectively by thrombin further enhances specificity of the thromboprophylaxis. Results of the recent animal studies give us a strong reason to believe that with further work, these novel targeted therapeutics will offer safe and effective means for management of thrombosis.

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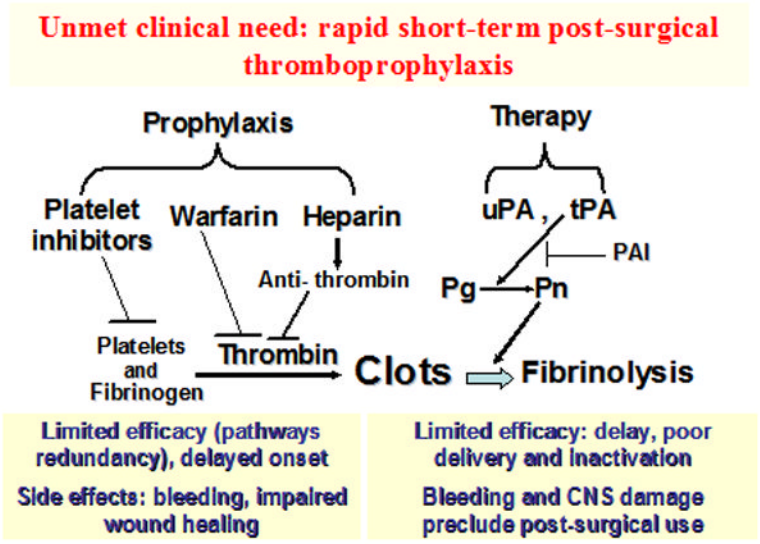

Figure 1. Insufficiencies of current anti-thrombotic agents

This schema illustrates current anti-thrombotic agents in use for either prophylaxis or treatment, and their limitations. Platelet inhibitors, warfarin and heparin are common prophylactic agents, these agents work to inhibit clot formation. However, these agents have limited efficacy, and severe adverse effects such as bleeding and impaired wound healing. In therapy, uPA is effective by directly inhibiting plasminogen, while tPA activates the conversion of plasminogen (Pg) to plasmin (Pn), which results in clot breakdown. Although, both of these agents have significant side effects, limited efficacy, such as, poor delivery, and bleeding complications. Abbreviations found within the figure- PAI, Plasminogen activator inhibitor; Pg, Plasminogen; Pn, Plasmin; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

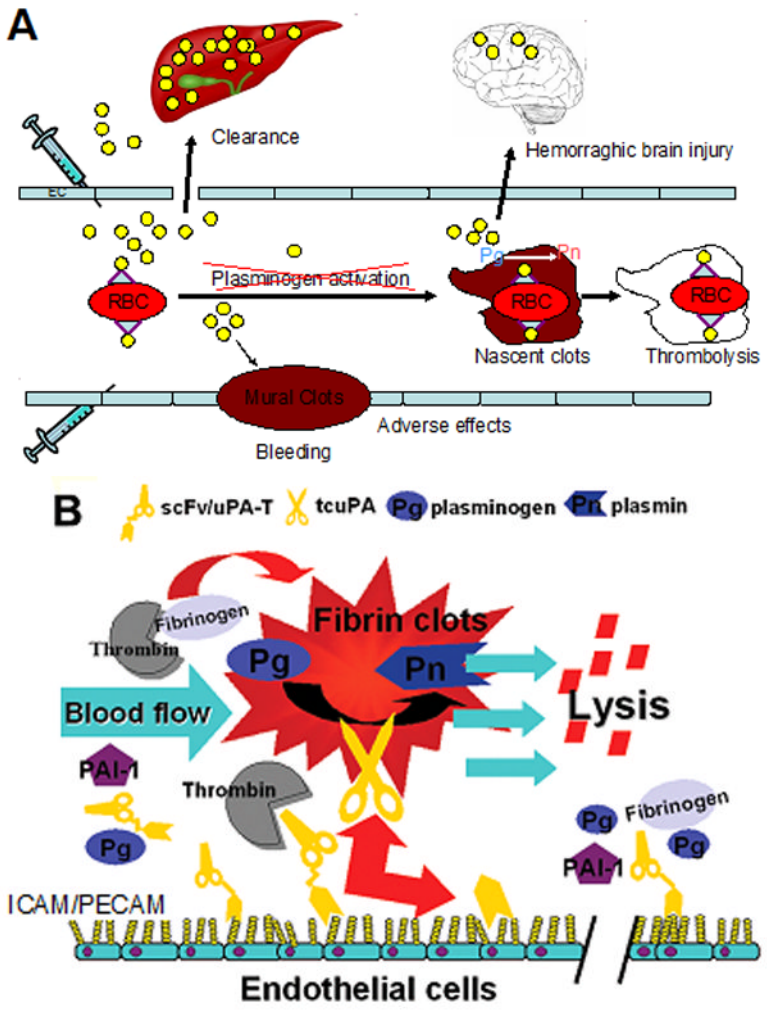

Figure 2. Strategies for coupling therapeutic agents to RBC and ECs

(A) Plasminogen activators (yellow dots) are relatively ineffective, in part due to rapid uptake by liver, and unsafe due to bleeding (indiscriminate lysis of hemostatic mural clots), vascular side effects (e.g. activation of receptors on endothelial cells, EC) and injurious effects of tPA diffusing into the CNS. Coupling to RBC will dramatically prolong the longevity of the scFv/tPA variant. RBC will restrain scFv/tPA binding to cellular receptors, and restrict its access into mural hemostatic clots and the CNS. Propulsion of RBC towards the mainstream will further offset interactions of the pro-drug with hemostatic clots and vascular walls. RBC-bound scFv/tPA will have virtually unlimited access to the interior of nascent pathological thrombi and thereby will dissolve pathological intravascular clots and prevent vascular occlusion. (B) As with RBC/tPA, ICAM or PECAM targeting enables one to target more locally in this case the pulmonary vasculature versus more systemically in the case of RBCs. By diversifying targeting, we can improve specificity and limit adverse effects. Abbreviations found within the figure-ICAM-1, Intercellular adhesion molecule, 1; PAI-1, Plasminogen activator inhibitor-1; PECAM-1, Platelet endothelial cell adhesion molecule-1; Pg, Plasminogen; Pn, Plasmin; RBC, red blood cell; tPA, tissue plasminogen activator; uPA-T, urokinase plasminogen activator-thrombin.

Table 1

Current anti-thrombotic agents

This table describes the current anti-thrombotic agents that are commonly/currently used in patients. Included are descriptions of how the respective drugs are delivered, their half-life, when it is used, how it works and any adverse effects.

Drug	Time/Onset of Action	Description
Warfarin	• 12 hour delay in action • Hypoprothrombinaemia 36–72 hours • Duration of action 4–5 days	• Vitamin K antagonist inhibits synthesis of coagulation factors • Standard care for long-term prophylaxis of coagulation • High risk of bleeding, dose monitoring is necessary.
Heparin	• IV: 30–60 seconds • SubQ: 20–30 minutes • Short half-life (~1 hour)	• Indirect thrombin and Factor Xa inhibitor (activates anti-thrombin III) • Current standard for emergency anticoagulation. • High risk of bleeding and heparin induced thrombocytopenia (HIT)
tPA	• IV: 30 seconds • Short half-life • IV bolus followed by Infusion	• Within 3 hours of event, or 6 hours, arterial catheter • Patients with early onset of stroke, PE, AMI • High risk of bleeding, neurotoxicity
SK	• Two half–lifes: fast ~18 mins and slower ~83 mins	• Fibrinolytic: thrombolytic therapy • Least expensive fibrinolytic agent, but is antigenic, also causes hypotension (dose related)
UK	• Short half-life (~20mins) • IV bolus followed by infusion	• Fibrinolytic agent more commonly used for peripheral intravascular thrombus and occluded catheters

Abbreviations found within the table-IV, intravenous; SK, streptokinase; SubQ, Subcutaneous; tPA, tissue plasminogen activator; UK, Urokinase; uPA, urokinase plasminogen activator; uPA-T, urokinase plasminogen activator-thrombin.

Table 2

RBC and EC targeted anti-thrombotic interventions

This table describes anti-thrombotic agents that have been targeted to different vascular targets. Included is the location of their targeting, influence on fibrinolysis and any other notable findings.

Drug	Target	Description
tPA	RBC	• ↑ Rate of fibrinolysis; lysing clots within and during clot extension • More effective even when 10-fold greater non-targeted tPA was used • Restored perfusion without hemorrhagic complications unlike non-targeted t-PA
uPA-T	RBC	• Caused thrombin-induced fibrinolysis • 35% of scFv/uPA-T was retained in the blood, 48hrs post-injection • A single IV injection of scFv/uPA-T provided effective prophylaxis against arterial • and venous thrombosis for up to 24 hours
tPA	ICAM-1	• Accumulation two orders of magnitude higher than IgG targeted control • ↑ Rate of fibrinolysis
uPA	PECAM-1	• Pulmonary endothelium accumulation for at least 3 hrs • ↑ Rate of fibrinolysis • Improved blood reperfusion without hemorhagic complications
uPA-T	PECAM-1	• Pulmonary endothelium accumulation for at least 3 hrs • More effective than uPA, resisting PAI-1 inhibition until thrombin activated • ↑↑ Rate of fibrinolysis • Significantly attenuated pulmonary fibrin depostion

Abbreviations found within the table- ICAM-1, Intercellular adhesion molecule, 1; PECAM-1, Platelet endothelial cell adhesion molecule-1; RBC, red blood cell; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPA-T, urokinase plasminogen activator-thrombin.