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REVIEW ARTICLE

Fibrinolysis: strategies to enhance the treatment of acute ischemic stroke

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Summary. Stroke is a major cause of disability worldwide, and is the second leading cause of death after ischemic heart disease. Until recently, tissue-type plasminogen activator (t-PA) was the only treatment for acute ischemic stroke. If administered within 4.5 h of symptom onset, t-PA improves the outcome in stroke patients. Mechanical thrombectomy is now the preferred treatment for patients with acute ischemic stroke resulting from a large-artery occlusion in the anterior circulation. However, the widespread use of mechanical thrombectomy is limited by two factors. First, only 10% of patients with acute ischemic stroke have a proximal large-artery occlusion in the anterior circulation and present early enough to undergo mechanical thrombectomy within 6 h; an additional 9–10% of patients presenting within the 6–24-h time window may also qualify for the procedure. Second, not all stroke centers have the resources or expertise to perform mechanical thrombectomy. Nonetheless, patients who present to hospitals where thrombectomy is not an option can receive intravenous t-PA, and those with qualifying anterior circulation strokes can then be transferred to tertiary stroke centers where thrombectomy is available. Therefore, despite the advances afforded by mechanical thrombectomy, there remains a need for treatments that improve the efficacy and safety of thrombolytic therapy. In this review, we discuss: (i) current treatment options for acute ischemic stroke; (ii) the mechanism of action of fibrinolytic agents; and (iii) potential strategies to manipulate the fibrinolytic system to promote endogenous fibrinolysis or to enhance the efficacy of fibrinolytic therapy.

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Introduction

Stroke is a major cause of death and disability worldwide [1,2]. Approximately 85% of strokes are ischemic in origin and are caused by blockage of blood flow to the brain, leading to irreversible brain injury and subsequent neurological deficits [3]. Rapid restoration of blood flow is essential to limit disability in patients with acute ischemic stroke. The only approved pharmacological treatment for restoration of blood flow is intravenous tissue-type plasminogen activator (t-PA) [4], which must be administered within 4.5 h of symptom onset. Unfortunately, with this narrow time window, the majority of patients with acute ischemic stroke do not receive such treatment [1,5–7]. Furthermore, even with t-PA administration, only ~15% of patients have complete recovery, and up to 3% have fatal or non-fatal intracranial hemorrhage.

Endovascular thrombectomy is now the standard of care for patients with acute ischemic stroke secondary to large-vessel anterior circulation occlusion who present within 24 h of stroke symptom onset. However, only ~20% of stroke patients have large-vessel occlusion, and delivering treatment to such patients within a rapid time frame is challenging, because the procedure can only be performed in highly specialized centers. Although patients can be given t-PA and then transferred to a tertiary-care center, a so-called 'drip and ship' approach, identifying appropriate patients for transfer is challenging in hospitals without access to advanced imaging techniques such as computed tomography perfusion scanning or magnetic resonance imaging. Although all patients with suspected large-vessel occlusion could be transferred, this strategy places a large burden on tertiary-care centers.

The expanded and varying time windows used across the thrombectomy trials creates opportunities and

challenges for the treatment of stroke patients with proximal occlusion. Patients with 'wake-up strokes', who are typically deemed to be ineligible for thrombolysis because of the unknown time of onset, may be candidates for endovascular therapy if proximal occlusion is identified. Multimodal imaging, including perfusion scanning and assessment of collaterals, helps to identify patients who may benefit from reperfusion despite the unknown time of onset. Such imaging has the potential to eliminate strict time windows for delivery of therapy, but the role of collaterals in patient selection and their impact on outcome remains to be determined. Therefore, questions remain.

Despite the advances made with endovascular thrombectomy, at most, only 20% of patients with acute ischemic stroke are eligible for the procedure. Furthermore, there is evidence that t-PA administration in conjunction with thrombectomy is more efficacious than thrombectomy alone [8–11]. Consequently, fibrinolytic therapy remains a mainstay of stroke treatment. Therefore, there remains a need for new strategies to enhance the efficacy and safety of thrombolytic therapy for acute ischemic stroke. The purposes of this article are to: (i) review the types of acute ischemic stroke; (ii) describe how t-PA initiates fibrinolysis; (iii) explore how variations in clot composition and structure may modulate the response to t-PA; and (iv) identify novel strategies for enhancing the efficacy and safety of fibrinolytic therapy for acute ischemic stroke.

Acute ischemic stroke

Ischemic stroke is divided into two major subtypes, thrombotic and embolic [3]. Thrombotic strokes, which account for ~45% of all ischemic strokes, can occur in the large or small vessels in the brain. Thrombotic strokes in large cerebral vessels usually occur when a thrombus forms on top of a disrupted atherosclerotic plaque. Small-vessel or lacunar infarcts occur when small vessels in the brain are occluded, often as a result of hypertension [12].

Embolic strokes account for ~20% of all ischemic strokes [3,13]. In this case, the thrombus originates outside of the brain, and then travels through the bloodstream and lodges in a cerebral artery. The heart is the most common source of such thrombi, particularly in patients with atrial fibrillation. Thrombi that form on top of disrupted atherosclerotic plaques in the aortic arch or in the carotid arteries can also embolize into the cerebral vessels.

Cryptogenic strokes constitute the remainder of acute ischemic strokes. A cryptogenic stroke is defined as an ischemic stroke that is not attributable to atrial fibrillation, large-artery atherosclerosis, or small-vessel thrombosis; the cause of the stroke may be uncertain because the neurological event was transient, because thorough investigations were not performed to investigate the source, or because the cause was truly unknown [14,15]. Approximately 25–30% of patients with cryptogenic stroke have subclinical atrial fibrillation that can be revealed with prolonged cardiac monitoring [16–18]. However, the lack of benefit of rivaroxaban over aspirin for secondary prevention in patients with cryptogenic stroke suggests that occult atrial fibrillation is not a major driver of stroke in such patients [19].

Regardless of the type of ischemic stroke, t-PA is the primary treatment. However, only a minority of patients have complete stroke resolution with t-PA therapy. Larger thrombi in the more proximal cerebral arteries are less susceptible to lysis than smaller, more distal, ones, which may reflect differences in the composition of the thrombi or the failure of t-PA to penetrate into larger clots [20–22].

Thrombus composition

All arterial thrombi are composed of aggregated platelets and fibrin, but the proportions differ according to the source of the thrombus. Thrombi that form in large arteries on top of disrupted atherosclerotic plaques are rich in platelets and contain relatively little fibrin. In contrast, thrombi that form in the left atrial appendage in patients with atrial fibrillation tend to be more organized, and contain large amounts of fibrin and fewer platelets [20–23].

The structure of the fibrin fibers within the thrombus may also influence its susceptibility to lysis. Clot structure can be influenced by the circulating levels of fibrinogen and by the concentrations of clot-promoting substances such as ions, inorganic polyphosphates, neutrophil extracellular traps, histones, and cell-free DNA [24–28]. Thrombi in stroke patients tend to contain thick fibrin fibers, which make them more porous and more susceptible to deformation than clots composed of thick, densely packed fibers [29]. Although the greater porosity of the thrombi in stroke patients should facilitate diffusion of t-PA into the interstices of the clot network [30], many such thrombi are resistant to lysis. This resistance may reflect elevations in the circulating concentrations of fibrinolytic inhibitors such as plasminogen activator inhibitor type 1 (PAI-1), α_2-antiplasmin, and thrombin-activatable fibrinolysis inhibitor (TAFI; also known as procarboxypeptidase U and procarboxypeptidase B2) [31–33].

With the advent of thrombectomy, it is now possible to examine the composition of thrombi extracted from the cerebral vessels of stroke patients [34]. Thrombi containing large amounts of calcium phosphate are stiff, and less amenable to extraction [35]. Consistent with their formation under low-shear conditions, thrombi extracted from patients with cardioembolic stroke are fibrin-rich, but also contain platelets and white blood cells [23]. In contrast, thrombi removed from patients with non-cardioembolic strokes contain more red blood cells, less fibrin and fewer

platelets than thrombi from patients with cardioembolic strokes [36]. Despite differences in composition according to the site of thrombus origin, there is no consistent correlation between thrombus histopathology and the etiology of stroke [37].

Fibrinolysis in stroke

Thrombolytic therapy with t-PA initiates endogenous fibrinolysis by converting the zymogen plasminogen to its active form, plasmin (Fig. 1) [38]. t-PA circulates in an active, single-chain form at low concentrations (~ 0.2 nM) [39], but shows increased functionality in the presence of fibrin [40–42]. Plasmin not only solubilizes fibrin but can also degrade fibrinogen, as well as factor (F) V, FVIII, FIX, FXI, and FXII, which can impair the hemostatic potential. Therefore, regulation of plasmin generation and activity is essential [39,43].

Plasminogen activation by t-PA is regulated by PAI-1. Although the high concentrations of t-PA administered for treatment of acute ischemic stroke exceed those of circulating PAI-1, PAI-1 released from activated platelets within occlusive thrombi may limit the local activity of t-PA [44]. Therefore, PAI-1 is one of the factors that renders thrombi resistant to t-PA.

Plasmin is inhibited by α_2-antiplasmin, a 63 kDa serpin that forms a covalent bond with the active site serine of plasmin, thus inactivating it [39]. Activated FXIII (FXIIIa), the transglutaminase that is essential for crosslinking fibrin fibers, also crosslinks α_2-antiplasmin onto circulating fibrinogen. TAFI, a third regulator of fibrinolysis, is a procarboxypeptidase that can also be crosslinked to fibrin by FXIIIa [45]. TAFI is a 60 kDa zymogen that is activated to activated TAFI (TAFIa) by plasmin, thrombin, and the thrombin–thrombomodulin complex [46]. TAFIa removes C-terminal lysine residues on fibrin (Fig. 1). TAFIa also indirectly modulates clot lysis by reducing plasmin binding to fibrin, thereby rendering plasmin susceptible to inhibition by circulating α_2-antiplasmin [46]. Therefore, inhibitors of α_2-antiplasmin, FXIIIa and TAFIa have the potential to enhance fibrinolysis.

Cerebral fibrinolysis

Role of t-PA in the brain

Although t-PA initiates intravascular fibrinolysis in the systemic circulation, t-PA modulates extracellular matrix degradation in the brain [47]. t-PA is synthesized by endothelial cells lining the cerebral blood vessels, and is highly expressed in the hippocampus, amygdala, cerebellum, and hypothalamus [47]. In the brain, t-PA augments activation of the complement system, and suppression of complement activation reduces cerebral edema and hemorrhage in animal models of stroke [48,49].

In patients with acute ischemic stroke, t-PA and plasmin generated in the brain may adversely affect neuronal function by: (i) compromising the blood–brain barrier (BBB) through activation of matrix metalloproteases (MMPs) that digest extracellular matrix proteins [50]; (ii) mediating cortical cell apoptosis by activating glutamate receptors [51]; and (iii) decreasing blood flow to the penumbra, thereby contributing to infarct extension [51]. To regulate these processes, astrocytes express PAI-1, and neurons secrete neuroserpin [38,47]. PAI-1 inhibits t-PA, whereas neuroserpin inhibits both t-PA and plasmin [47]. Therefore, the brain has the capacity to modulate fibrinolytic activity.

Recent advances in our understanding of how the BBB is disrupted in patients with stroke has focused mainly on the role of MMPs. Plasma levels of MMPs increase in patients with acute ischemic stroke. The most important MMP in the brain is MMP-9, a latent protein that is activated by t-PA or plasmin [52]. Once activated, MMP-9 degrades extracellular matrix components such as collagen, laminin, and fibronectin, thereby increasing the permeability of the BBB, which can lead to cerebral edema, infarct extension, and intracerebral hemorrhage [53]. In mouse models of stroke, MMP-9 is responsible for many of the deleterious effects of t-PA [54], although MMP-2 may also be involved [55]. Recent studies have suggested that sirtunin 5 contributes to breakdown of the BBB in animal models of stroke. Sirtunin 5 is expressed by white blood cells, which are abundant in thrombi from patients with cardioembolic stroke, and increases BBB permeability by degrading occludin, an important tight-junction protein [56]. Although strategies to prevent breakdown of BBB permeability by inhibitors of MMPs and sirtunin 5 have shown promise in animal models of stroke, their utility in humans is unknown.

Plasmin generation in the brain

Plasminogen activation plays a key role in the central nervous system by modulating synaptic plasticity, neuronal inflammation, and degeneration. Data suggest that plasminogen is expressed in the cortex, hippocampus, and cerebellum [47,57], but it may also enter the brain by crossing the BBB. Astrocytes provide a surface onto which plasminogen and t-PA (but not urokinase plasminogen activator) bind, thereby enhancing their interaction [58]. In addition to stimulating inflammation and BBB disruption, plasmin in the brain degrades laminin, augments neuronal detachment, and promotes endothelial and smooth muscle cell apoptosis; all of these activities increase the risk of brain hemorrhage [58]. To regulate this process, astrocytes take up surface-bound plasminogen and plasmin via endocytosis, and target them to lysosomes for degradation [58]. In animal models of stroke, the neurotoxic effects of plasmin are attenuated by *N*-

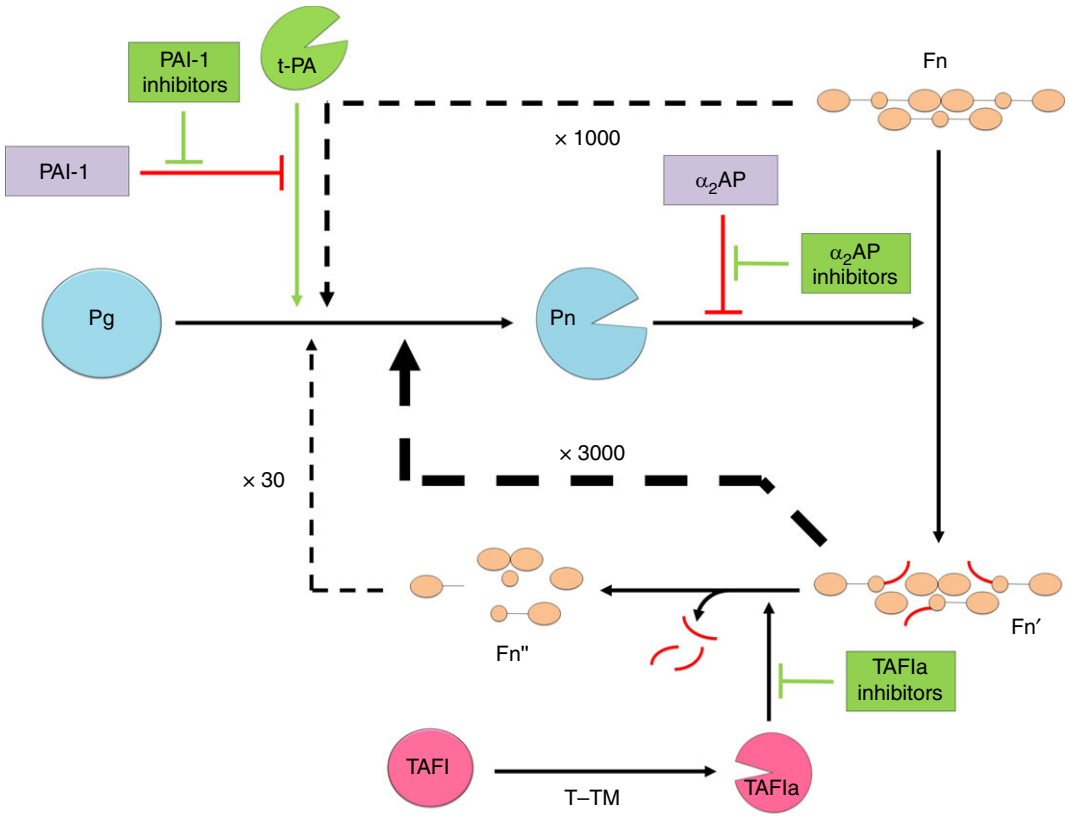

Fig. 1. The current targets of interest within the fibrinolytic system for improving thrombolytic therapy. Damaged endothelial cells release tissue-type plasminogen (Pg) activator (t-PA), which cleaves the activation sequence (Arg561–Val562) on Pg to generate plasmin (Pn). Fibrin (Fn) that is generated during clot formation acts as a cofactor during its own breakdown by enhancing Pg activation. It does so by acting as a template that facilitates the binding of both Pg and t-PA to its surface. Pn that is initially generated initiates proteolytic cleavage of Fn to generate plasmin-modified Fn (Fn′), which contains newly exposed C-terminal lysine residues (red curves). The presence of these C-terminal lysine residues allows for the binding of additional Pg and t-PA, which results in further enhancement of Fn cofactor activity. These C-terminal lysine residues on Fn′ are then susceptible to proteolysis by activated thrombin-activatable fibrinolysis inhibitor (TAFIa), to generate TAFIa-modified fibrin (Fn″), which downregulates the cofactor activity of Fn for Pg activation. Fibrinolysis is regulated by the serpins α_2-antiplasmin (α_2AP) and Pg activator inhibitor type 1 (PAI-1), which downregulate Pn activity and Pg activation, respectively. Thrombin-activatable fibrinolysis inhibitor (TAFI) activation is mostly mediated by the thrombin–thrombomodulin complex (T–TM). The dotted lines represent the cofactor activity of Fn and its derivatives. The solid red lines indicate inhibition. The green lines and text boxes indicate current targets of interest for improving thrombolytic therapy: (i) enhance t-PA activity and/or safety; and (ii) inhibit PAI-1, α_2AP and TAFIa to promote fibrinolysis. [Color figure can be viewed at wileyonlinelibrary.com]

methyl-D-aspartate receptor antagonism and neuroserpin [59,60]. However, the role of these agents in patients with stroke is unknown.

Strategies to enhance fibrinolytic therapy for acute ischemic stroke

Several strategies are under investigation to augment fibrinolysis in patients with acute ischemic stroke or to enhance safety. These include: (i) novel delivery strategies; (ii) the use of fibrinolytic agents that may be more effective and safer than t-PA; (iii) attenuating the activity of the major regulators of fibrinolysis, including PAI-1, α_2-antiplasmin, FXIIIa, and TAFIa; and (iv) the use of activated protein C variants to attenuate the complications of t-PA therapy. Each of these strategies will be briefly discussed.

Novel delivery strategies

Large proximal cerebral thrombi are particularly resistant to lysis by intravenous t-PA. Initial attempts to overcome this problem focused on combining intravenous t-PA delivery with intra-arterial delivery. In pilot studies, recanalization rates were higher with intra-arterial t-PA delivery than with intravenous delivery, but neurological outcomes were not improved. A subsequent study evaluated initial treatment with intravenous t-PA followed by intra-arterial t-PA delivery [61]. Although the results obtained with this combination regimen were promising, this approach was abandoned when endovascular thrombectomy became the treatment of choice for acute ischemic stroke caused by proximal thrombi [8–11].

Other experimental methods to enhance t-PA delivery or activity include the use of nanocarriers [62] and

microbubbles combined with ultrasound [63]. Examples of nanocarriers include liposomes and polymer-based or magnetic nanoparticles. These encapsulate t-PA or allow t-PA binding to its outer layer of the liposome lipid bilayer or the inorganic shell of nanoparticles. In stroke models in animals, encapsulating t-PA within nanocarriers or binding of it to their surfaces enhances fibrinolysis by protecting t-PA from inhibition by circulating PAI-1, and by increasing the capacity of t-PA to permeate into the clot [62].

Microbubbles are gas bubbles that can be fragmented into smaller bubbles in response to oscillations caused by externally applied ultrasound. Generation of these fragmented microbubbles at the clot surface helps to disrupt the clot, thereby enhancing the activity of intravenous t-PA. Although, in one study, t-PA in combination with microbubbles resulted in more rapid recanalization in patients with acute ischemic stroke than t-PA alone [64], additional trials are needed to confirm these results. By coating of microbubbles with lipid or albumin and binding t-PA to the surface coating, microbubbles can be used as nanocarriers [62]. The efficacy of microbubble nanocarriers relative to other nanocarriers of t-PA, however, requires further investigation.

Ultrasound enhances the fibrinolytic activity of t-PA because oscillation forces mechanically disrupt the thrombus, thereby promoting the penetration of t-PA [63]. In patients with acute ischemic stroke, application of intravascular Doppler ultrasound in patients given intravenous t-PA augmented recanalization of the occluded cerebral artery in one study [65]. Ultrasound can also be used in conjunction with microbubbles as drug carriers (echogenic liposomes) [66,67]. Consisting of a phospholipid bilayer shell, these liposomes encapsulate or bind t-PA or plasmin on their surface and incorporate gas microbubbles within their aqueous core. Application of ultrasound at the site of the occlusive thrombus induces expansion of the microbubbles, thereby disrupting the liposomes. The released microbubbles promote the penetration of the t-PA or plasmin carried by the liposomes into the thrombus, thereby increasing fibrinolytic capacity. In animal models of thrombosis, ultrasound-controlled delivery with this nanotechnology protects the bound t-PA or plasmin from inhibition by circulating PAI-1 and α_2-antiplasmin, respectively, and localizes their activity to the thrombus, which may enhance fibrinolysis and reduce the risk of bleeding by attenuating systemic plasmin generation or plasmin activity [67]. Although this technology is promising, studies in humans are needed to assess its utility.

More effective and safer fibrinolytic agents

Ongoing studies are comparing tenecteplase (TNK-tPA) [68,69] with t-PA for the treatment of patients with acute ischemic stroke. TNK-tPA is easier to administer because it has a half-life that is 8.5-fold longer than that of t-PA. Consequently, TNK-tPA can be given as a single intravenous bolus, whereas t-PA must be given as a bolus followed by an infusion. One phase II study showed a higher recanalization rate at 24 h with TNK-tPA than with t-PA [70], and another showed that the risk of bleeding complications was lower with TNK-tPA than with t-PA [68]. Despite these promising early results, TNK-tPA offered no benefit over t-PA in the phase III Norwegian Tenecteplase Stroke Trial (NOR-TEST) [71]. In this trial, 1107 patients with ischemic stroke who presented within 4.5 h of symptom onset were randomized to receive TNK-tPA (0.4 mg kg^{-1} intravenous bolus) or t-PA (0.9 mg kg^{-1} with 10% as an intravenous bolus and the remainder as an infusion over a period of 60 min). Patients with an increased bleeding risk and those with a baseline modified Rankin Scale (mRS) score of ≥ 3 were excluded. The incidence of the primary endpoint – an excellent outcome (mRS score of 0–1) at 3 months – did not differ significantly between the two groups. The secondary outcome of neurological improvement (as measured by use of the National Institutes of Health Stroke Scale score) was also similar in the two groups. Although additional trials with TNK-tPA are underway, the disappointing results of NOR-TEST make it unlikely that TNK-tPA will offer substantial benefits over t-PA for the treatment of patients with acute ischemic stroke.

FXIIIa inhibitors

FXIIIa renders thrombi resistant to lysis by crosslinking fibrin monomers, and also by crosslinking α_2-antiplasmin and TAFIa onto the fibrin surface. Therefore, inhibitors of FXIIIa have the potential to enhance fibrinolysis. However, even small amounts of FXIIIa are sufficient for this crosslinking activity. Therefore, near-complete inhibition of FXIIIa would be required to modulate fibrinolysis. This is problematic, because homozygous FXIII deficiency is characterized by a bleeding diathesis, which includes intracranial bleeding. Furthermore, it is difficult to develop inhibitors of FXIIIa that do not cross-react with tissue transglutaminases. Therefore, this approach has not yet been evaluated in humans.

PAI-1 inhibitors

Circulating PAI-1 and PAI-1 released from activated platelets within and surrounding the thrombus inhibit t-PA, thereby limiting its effectiveness. Numerous PAI-1 inhibitors have been described, including antibodies, nanobodies, and small molecules [72–75]. Although studies with many of these agents have yielded promising results in preclinical models, none has been tested in patients with acute ischemic stroke.

α_2-Antiplasmin inhibitors

α_2-Antiplasmin is the major inhibitor of plasmin, and α_2-antiplasmin crosslinked to fibrin and circulating α_2-antiplasmin limit clot digestion. Circulating levels of α_2-antiplasmin are higher than those of PAI-1 (1 μM and 0.5 nM, respectively), which poses a challenge for inhibitor development. Furthermore, inhibition of circulating α_2-antiplasmin may lead to bleeding if unopposed plasmin degrades fibrinogen and other clotting factors [75]. Nonetheless, inhibitory antibodies against α_2-antiplasmin have been developed. Such antibodies promote fibrinolysis in animal models [76], but have not been evaluated in humans.

TAFIa inhibitors

TAFIa inhibitors enhance clot lysis by: (i) promoting feedback activation of plasminogen by t-PA; and (ii) enabling plasmin to remain bound to fibrin, where it is protected from inhibition by α_2-antiplasmin. Small-molecule TAFIa inhibitors such as potato tuber carboxypeptidase inhibitor (PTCI) [77] and 2-guanidinoethylmercaptosuccinic acid [78] promote t-PA-mediated fibrinolysis *in vitro*, and administration of PTCI in conjunction with t-PA promoted fibrinolysis without increasing bleeding in rabbit thrombosis and ear bleeding models, respectively [79]. Small-molecule TAFIa inhibitors show biphasic activity. Thus, they inhibit TAFIa when given in high concentrations, and promote TAFIa activity at low concentrations. This phenomenon can be explained by the fact that, when administered in low concentrations, the small molecules bind to TAFIa and stabilize it, thereby attenuating its capacity to undergo autodenaturation. In contrast, when the small molecules are administered in high concentrations, this stabilizing effect is overcome by complete TAFIa inhibition.

Despite promising results in preclinical models [80], the development of aminopyridine mercaptane (AZD9684), a small-molecule TAFIa inhibitor, was halted when a phase II study in patients with pulmonary embolism revealed little or no activity. DS-1040 [81], a second-generation small-molecule TAFIa inhibitor, produced dose-dependent inhibition of TAFIa when administered to healthy volunteers. Ongoing placebo-controlled phase II studies are evaluating DS-1040 as an adjunct to anticoagulant therapy in patients with submassive pulmonary embolism (NCT02923115) and in patients with acute ischemic stroke who are ineligible for t-PA because they present beyond the 4.5-h time window (ASSENT, NCT02586233).

Variants of activated protein C

In addition to its capacity to downregulate coagulation by inactivating activated FV (FVa) and FVIIIa, activated protein C also has cytoprotective effects, because it can bind to the endothelial protein C receptor and induce cell signaling via PAR-1 activation. The cytoprotective effect of activated protein C is independent of its anticoagulant activity [82]. This finding prompted the development of variants of activated protein C that have minimal anticoagulant activity but retain their cytoprotective effects. One such variant, designated 3K3A-APC, reduced brain damage in an animal model of traumatic brain injury [83]. The potential neuroprotective effects of this agent are now being evaluated in a phase II trial (RHAPSODY, NCT02222714) in patients with acute ischemic stroke. Therefore, 3K3A-APC or other activated protein C variants may provide a novel approach to limiting the neurotoxic effects of t-PA and plasmin.

Conclusions

Despite the introduction of endovascular thrombectomy, ischemic stroke remains a major cause of death and disability worldwide. A better understanding of the molecular mechanisms of fibrinolysis and new insights into the cytoprotective effects of activated protein C have resulted in the development of new agents that have the potential to promote endogenous fibrinolysis and enhance the activity of t-PA or to modulate the neurotoxic effects of t-PA and plasmin. The most promising of these strategies include novel t-PA delivery systems, inhibitors of TAFIa, and variants of activated protein C. Well-designed clinical trials are needed to determine whether these novel strategies will reduce morbidity and mortality in patients with acute ischemic stroke.

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