Cytoprotective-selective activated protein C therapy for ischaemic stroke

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Summary
Despite years of research and efforts to translate stroke research to clinical therapy, ischaemic stroke remains a major cause of death, disability, and diminished quality of life. Primary and secondary preventive measures combined with improved quality of care have made significant progress. However, no novel drug for ischaemic stroke therapy has been approved in the past decade. Numerous studies have shown beneficial effects of activated protein C (APC) in rodent stroke models. In addition to its natural anticoagulant functions, APC conveys multiple direct cytoprotective effects on many different cell types that involve multiple receptors including protease activated receptor (PAR) 1, PAR3, and the endothelial protein C receptor (EPCR). Application of molecular engineered APC variants with altered selectivity profiles to rodent stroke models demonstrated that the beneficial effects of APC primarily require its cytoprotective activities but not its anticoagulant activities. Extensive basic, preclinical, and clinical research provided a compelling rationale based on strong evidence for translation of APC therapy that has led to the clinical development of the cytoprotective-selective APC variant, 3K3A-APC, for ischaemic stroke. Recent identification of non-canonical PAR1 and PAR3 activation by APC that give rise to novel tethered-ligands capable of inducing biased cytoprotective signalling as opposed to the canonical signalling provides a mechanistic explanation for how APC-mediated PAR activation can selectively induce cytoprotective signalling pathways. Collectively, these paradigm-shifting discoveries provide detailed insights into the receptor targets and the molecular mechanisms for neuroprotection by cytoprotective-selective 3K3A-APC, which is currently a biologic drug in clinical trials for ischaemic stroke.

Keywords
Stroke, activated protein C, PAR1, PAR3, EPCR

Introduction
Stroke is a major cause of death and disability worldwide. In 2010, stroke caused 5.8 million deaths worldwide, which is 11.1% of all deaths globally (1). Ischaemic stroke is highly prevalent (87%), whereas intracranial haemorrhage and subarachnoid haemorrhage strokes account for 10% and 3%, respectively. In the United States, someone suffers a stroke approximately every 40 seconds, resulting in a death every 4 minutes (min) (2, 3). The current mortality rate ~16% points to the true health burden of stroke, as most strokes are not fatal (2–4). Surviving stroke is the most common cause of neurological disability. One third of stroke survivors remain functionally disabled after one year and require ongoing support with significant adverse impact on the quality of life thereafter (2, 5). Serious long-term disability is the major socio-economic burden of stroke, e.g. it costs the US an estimated $36.5 billion each year of which 56% ($20.6 billion) is in direct medical costs (2). Improving the quality of life after stroke is therefore an unmet medical and socio-economic need. Here we take note of thrombolytic therapy for ischaemic stroke and review the promising possibility of cytoprotective-selective activated protein C (APC) for ischaemic stroke therapy.

Tissue-type plasminogen activator therapy for ischaemic stroke
Therapeutic treatment options for ischaemic stroke are limited and focus mainly on achieving early reperfusion by either thrombolytic or endovascular therapy (6). Recombinant tissue-type plasminogen activator (tPA; Alteplase) was approved over a decade ago for thrombolytic therapy and remains the only approved therapeutic (6). Because tPA therapy is effective only within an early window after ischaemic stroke (<4.5 hours [h]), therapy is limited to ~5% of stroke patients plus concerns for haemorrhagic conversion and tPAs neurotoxicity have limited its widespread application (2, 7–10).
Development of ischaemic stroke and its consequent damages are temporally and spatially variable. Early reperfusion reduces mortality and promotes improved functional outcome (6, 11). Surrounding the infarct core macroscopically, the penumbra initially escapes acute cell death due to some collateral blood flow but is at risk of irreversible damage when hypoperfusion persists and is prone to reperfusion-associated injury (12). The time-limited benefit of tPA therapy is consistent with the concept that the ischaemic damage to the penumbra is reversible for a limited time (6, 13). Although more aggressive thrombolytic therapy might seem beneficial, tPA conveys increased risk for haemorrhagic transformation and for neurotoxicity. Hence, there is a delicate balance between tPAs beneficial and harmful effects (9). Despite the risk for haemorrhagic conversion, an extension of the window for tPA therapy from 3 h to 4.5 h has been approved in many countries and recommended by the American Heart Association although not yet supported by the Food and Drug Administration (FDA) (13). To increase the efficacy and safety of tPA, neuroprotective adjunctive agents that blunt tPAs haemorrhagic conversion and tPAs neuronal toxicity could have major potential advantages. Indeed, multifunctional APC is a remarkable neuroprotectant and its multiple actions plus its cellular and molecular mechanisms are reviewed below.

**Protein C and APC in stroke patients**

Protein C a vitamin K–dependent plasma glycoprotein and is one of the body’s natural anticoagulants circulating at 70 nmol/l as an inactive protease zymogen (14, 15). Physiologic generation of APC occurs on the endothelial surface by binding of thrombin to thrombomodulin and recruitment of protein C to the surface via binding to the EPCR (Figure 1) (14, 16). APC has potent anticoagulant activity due to its ability to inactivate factors Va and VIIIa, as well as multiple direct effects on vascular cells that are independent of its anticoagulant activity (14, 17). These direct effects on cells that include cell-signalling effects are collectively referred to as APC cytoprotective activities and generally include anti-apoptotic and anti-inflammatory activities, alterations of gene expression profiles and protection of endothelial barrier function. Most studies support the prevailing paradigm for APC’s direct cytoprotective actions on cells that are initiated by APC-mediated PAR1 activation when APC binds to EPCR localized in caveolin-1 enriched lipid rafts (14, 17–19). Depending on cell type, additional receptors may contribute to APC-initiated signalling such as sphingosine-1-phosphate receptor 1 (S1P1), apolipoprotein E receptor 2 (ApoER2), glycoprotein Ib, CD11b/CD18 (αMβ2; Mac-1), PAR3, and Tie2 (17, 20).

Multiple lines of evidence suggest that the brain is particularly sensitive to the activities of the APC anticoagulant and cytoprotective pathways. Infants born with severe protein C deficiency are often blind and develop mental retardation (21). Mice with genetically engineered protein C deficiency demonstrate selective fibrin deposition in brain blood vessels (22). Similar characteristic vasculature-specific local thrombosis is observed in mice carrying Factor V̄′̄Leiden (R506Q) but not in mice with a deficiency of ATIII, which form fibrin deposits in the heart and liver, but not in the brain (23, 24). Thus, characteristic brain-specific lesions seem to be related to a loss of specific, possibly APC-mediated cytoprotective functions in the brain rather than a general loss of natural anticoagulant activity.

Endogenous APC is part of a systemic anticoagulant and anti-inflammatory surveillance system and small levels of circulating levels of APC (~40 pmol/l) are normally found in the circulation. APC is also generated in the human brain, e.g. during the short is-
The neuroprotective effects of APC, defined as APC’s cytoprotective effects on cells of the neurovascular unit, were studied in conjunction with tPA therapy in rodent stroke models. Despite promoting restoration of cerebral blood flow and reducing fibrin deposition, tPA treatment increased haemorrhage after MCAO, increased injury volume, and decreased motor scores (37–39). When administered with or following tPA, APC reduced the tPA-mediated increases in injury volume, oedema, and neurologic motor scores, but also brought out the tPA’s reperfusion benefits as the tPA-APC combination was superior compared to either agent alone (37–39). Remarkably, APC also decreased tPA-induced brain microhaemorrhages (Figure 2). Thus, it appears that APC’s neuroprotective effects integrate well with reperfusion effects of tPA to improve outcome in ischaemic stroke models.

**APC activities and activity-selective APC variants**

Prevention of tPA-induced bleeding by APC required PAR1 since APC did not prevent tPA-induced bleeding in PAR1⁻/⁻ mice (38). In addition to PAR1, APC neuroprotective effects also required EPCR and PAR3 (34, 36, 37). Although genetically modified mice and receptor blocking antibodies clearly indicate important contributions of the cytoprotective pathway for neuroprotective effects of APC, activity-selective APC mutants provided additional answers to the question of the relative contribution of APC’s anticoagulant vs cytoprotective activities.

Engineering approaches for APC exploited the premise that the enzymatic substrates (factors Va and VIIIa vs PAR1/PAR3) and the
cofactors (phospholipids/protein S vs EPCR) for APC’s anticoagulant and cytoprotective activities differ in structure and function (Figure 1). A positively charged extended surface involving multiple polypeptide loops on APC is essential for interactions with its substrate, factor Va (Figure 3) (14, 40, 41). Since at least part of this factor Va exosite on APC is not involved in interactions with PAR1, targeting specific positive residues for mutation to alanine decreased anticoagulant activity yet retained normal cytoprotective activities (Table 1). Cytoprotective-selective APC mutants include RR229/230AA-APC, 3K3A-APC (KKK191–193AAA), 5A-APC (the combination of 3K3A-APC with RR229/230AA-APC), R193E-APC, and Cys67-Cys82-APC (R222C/D237C) (42–46). Other cytoprotective-selective APC mutants targeted the interaction with protein S in the middle and the protease domain at the top with the “active site” residues noted in red. On the top of the model, blue highlights five basic residues (KKK191–193 and RR229/230) which form a large positively charged exosite that recognises factor Va; mutations of these residues reduce anticoagulant activity but not cytoprotective activity. Purple highlights two residues (R222 and D237) which when mutated to Cys can form a disulfide bond, causing loss of most anticoagulant activity but retention of cytoprotective activity. Light green highlights the L38D mutation that reduces anticoagulant activity due to reduced protein S enhancement. Anticoagulant-selective APC mutants include mutations of E330 and E333 to Ala (orange) that selectively reduce PAR1 signalling, the E149A mutation (yellow) in the C terminus of the light chain, and mutation of L8 in the GLA-domain (dark green) that selectively disrupts APC binding to EPCR but not to negatively charged phospholipids. The model is based on the x-ray crystallographic structure of APC (1AUT) (90).

Although human APC is neuroprotective in the mouse (33, 34, 36), effective concentrations are much higher compared to effective murine APC concentrations (52). 3K3A-APC, 5A-APC, and E149A-APC were made as homologous mouse APC variants for in vivo proof of concept studies (Table 1) (51, 53). Murine cytoprotective-selective 5A-APC reduced infarct volume and oedema, and improved motor score after MCAO, whereas murine anticoagulant-selective E149A-APC worsened outcomes and increased brain haemorrhage after MCAO (54). Murine 3K3A-APC with 80% reduced anticoagulant activity provides 1.5-fold to two-fold enhanced neuroprotective effects compared to mouse wt-APC (55, 56). Human cytoprotective-selective APC mutants, as well as the murine variants, are neuroprotective in ischaemic stroke (57–59). Thus, the cytoprotective effects of APC provide neuroprotection in ischaemic stroke, whereas the anticoagulant effects of APC exacerbate injury and cause bleeding. These conclusions are consistent with results in experimental sepsis models where murine cytoprotective-selective APC variants (229/230, 3K3A, and 5A) reduce mortality, whereas E149A-APC can increase mortality (51, 53, 60).

The PAR paradox: APC vs thrombin

The neuroprotective effects of APC require EPCR-assisted activation of the GPCRs, PAR1 and PAR3 (36, 56). Yet thrombin activates these receptors much more efficiently, thereby stimulating neuronal damage and severe neurovascular injury (61, 62). These discordant effects of PAR1 activation by thrombin vs APC raises the question how PAR1 activation by different proteases can result in opposite effects? The PARs are unique in that they carry their own encrypted ligand encoded in the extracellular N-terminal tail (63). Proteolysis creates a new N-terminus that acts as a tethered-ligand for activation of the PAR. Thrombin activates PAR1 by proteolysis at canonical Arg41, whereas APC but not thrombin can activate PAR1 by proteolysis at non-canonical Arg46 (63, 64). Proteolysis at canonical vs non-canonical sites gives rise to different N-terminal sequences, i.e. different tethered ligand agonists, which begin at residue 42 (SFFLRN… or at residue 47 (NPNDKYE…). Similar to the N-terminal tethered agonists, synthetic peptides such as S/TFLLRN… (aka TRAP) or NPNDKYE… (aka TR47) elicit cell-signalling effects that resemble thrombin or APC effects, respectively.

Emerging insights into mechanisms for biased signalling of GPCRs, the requirement for β-Arrestin 2 for APC-induced cytoprotective PAR1 signalling, and PAR1 cleavages at Arg41 or Arg46 were integrated to provide a new paradigm for PAR1-mediated biased signalling (17, 64–68). As indicated (Figure 4), canonical and non-canonical PAR1 activation by different proteases generate biased tethered-ligands that differentially induce distinct active receptor conformations linked to unique signalling pathways. Cleavage by thrombin or a synthetic TRAP stabilizes PAR1 conformations that preferentially associate with G-proteins and induce MAPK phosphorylation, RhoA activation, and endothelial barrier disruptive effects. Cleavage by APC or a synthetic TR47 peptide stabilizes a different subset of PAR1 conformations that
preferentially employ biased β-Arrestin-mediated signalling that results in phosphorylation of Akt, activation of Rac1 and endothelial barrier protective effects. The functional selectivity between the PAR1 biased ligands, TRAP and TR47, on endothelial cells is remarkable as exemplified by the difference in MAPK phosphorylation vs Akt phosphorylation and RhoA vs Rac1 activation elicited by TRAP and TR47, respectively (64).

By mechanisms that are not yet entirely clear, PAR3 may modulate PAR1-dependent signalling and APC’s neuroprotective actions (36, 56, 69–72). Proteolysis of human PAR3 by APC occurs at non-canonical Arg41 thereby generating the PAR3 tethered ligand sequence GAPPNS… as opposed to thrombin generating the tethered ligand sequence TFRGAP… by proteolysis at canonical Lys38 (70). The different N-terminal peptide sequences generated from non-canonical cleavages of PAR1 and PAR3 by APC are biased peptide agonists that promote vascular integrity (64, 70). Collectively, new paradigms for biased PAR1 and PAR3 signalling due to non-canonical activation provide mechanisms for how different proteases mediate functional selectivity and why APC

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**Table 1: Activity-selective APC mutants.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Mutation(s)</th>
<th>Target</th>
<th>Anticoagulant activity</th>
<th>Cytoprotective activity</th>
<th>Selectivity ratio</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytoprotective-selective APC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3K3A-APC</td>
<td>KKK191–193AAA¶</td>
<td>FVa</td>
<td>4.6 % (25 %)§</td>
<td>114 % (100 %)†</td>
<td>25× (4x)</td>
<td>(44)</td>
</tr>
<tr>
<td>229/230-APC</td>
<td>RR229/230AA</td>
<td>FVa</td>
<td>13 % (33 %)</td>
<td>94 % (100 %)</td>
<td>7× (3x)</td>
<td>(44)</td>
</tr>
<tr>
<td>5A-APC</td>
<td>KKK191–193AAA + RR229/230AA</td>
<td>FVa</td>
<td>&lt;3 % (&lt;8 %)</td>
<td>100 % (100 %)</td>
<td>&gt;33× (&gt;13x)</td>
<td>(45)</td>
</tr>
<tr>
<td>Cys67-Cys82-APC</td>
<td>RD222/237CC</td>
<td>FVa</td>
<td>&lt;1 %</td>
<td>~50 %</td>
<td>&gt;50×</td>
<td>(42)</td>
</tr>
<tr>
<td>K193E</td>
<td>K193E</td>
<td>FVa</td>
<td>4 %</td>
<td>200 %</td>
<td>50×</td>
<td>(46)</td>
</tr>
<tr>
<td>APC-L38D</td>
<td>L38D</td>
<td>PS</td>
<td>&lt;5 %†</td>
<td>100 %</td>
<td>&gt;20×</td>
<td>(47)</td>
</tr>
<tr>
<td>APC-L38D/N229Q</td>
<td>L38D/N229Q</td>
<td>PS+PAR1</td>
<td>&lt;5 %</td>
<td>500 %</td>
<td>&gt;100×</td>
<td>(48)</td>
</tr>
<tr>
<td><strong>Anticoagulant-selective APC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E149A-APC</td>
<td>E149A</td>
<td>PS+?</td>
<td>420 % (400 %)</td>
<td>&lt;6 % (&lt;6 %)</td>
<td>&gt;70× (&gt;67x)</td>
<td>(51)</td>
</tr>
<tr>
<td>APC-E167A</td>
<td>E330A</td>
<td>PAR1</td>
<td>100 %</td>
<td>&lt;1 %</td>
<td>&gt;100×</td>
<td>(50)</td>
</tr>
<tr>
<td>APC-E170A</td>
<td>E333A</td>
<td>PAR1</td>
<td>100 %</td>
<td>&lt;1 %</td>
<td>&gt;100×</td>
<td>(50)</td>
</tr>
<tr>
<td>L8W-APC</td>
<td>L8Q</td>
<td>EPCR</td>
<td>~175 %†</td>
<td>~10 %†</td>
<td>~18×</td>
<td>(46)</td>
</tr>
</tbody>
</table>

¶ Numbering based on mature protein C. For conversion to chymotrypsin numbering, see Mather et al. (90). § Denoted are values for human APC in human plasma systems and in parentheses the corresponding values for the homologous murine APC mutants in murine plasma systems if data are available. † The percentages reflect the activity of APC mutants based on changes in the dose-response to achieve equal effects compared to wt-APC. ‡ Based on extrapolated values. PS is an abbreviation for protein S.
conveys neuroprotection via PAR1, whereas thrombin mediates neuronal damage and severe neurovascular injury via PAR1 (30, 61, 62).

Mechanisms of neuroprotection by APC

To provide neuroprotective effect on neurons and other brain cells inside and beyond the neurovascular unit, EPCR enables APC transport across the blood–brain-barrier (BBB) (73, 74). Inside the neurovascular unit, APC conveys multiple beneficial effects directly on neurons that include inhibition of apoptosis, anti-inflammatory effects, and alteration of gene expression profiles. As depicted in Figure 5, APC neuroprotective effects and its ability to prevent tPA-induced neurotoxicity in the neurovascular unit include: (i) protection of the BBB integrity, (ii) anti-inflammatory and anti-apoptotic effects on brain endothelial cells, and (iii) anti-apoptotic effects on neurons and other brain cells.

Protection of vascular integrity of the BBB by APC likely involves EPCR-dependent non-canonical activation of PAR1 and PAR3 that results in β-Arrestin 2-mediated biased signalling involving scaffolding by disheveled-2, and activation of Akt and Rac1. Activation of Rac1 in endothelial cells preserves cytoskeleton-mediated cell-cell interactions and prevents actin stress fibre formation (17, 64, 65). Additional contributions are possibly provided by APC-mediated transactivation of the Angiopoietin/Tie2 system and transactivation of the sphingosine-1-phosphate (S1P) receptor-1 (S1P1) involving APC-mediated activation of sphingosine kinase 1 and generation of S1P (14, 17). Furthermore, APC inhibits hypoxia-induced apoptosis in brain endothelial cells by blunting hypoxia-induced up regulation of pro-apoptotic p53 and Bax and preventing loss of anti-apoptotic Bcl-2 (34).

Prevention of tPA-induced haemorrhagic transformation by APC involves the entire neurovascular unit with multiple targets on multiple cells. Exact mechanisms for haemorrhagic transformation by tPA remain unknown but likely relate to non-thrombolytic functions of tPA that encompass: (i) disruption of the BBB, (ii) activation of matrix metalloproteases (MMP) especially MMP9, (iii) excitotoxic stimulation of N-methyl-D-aspartate (NMDA) receptors on neurons, and (iv) dilation of the cerebral vasculature in non-ischaemic areas thereby decreasing blood flow to the penumbra (7, 75, 76). A prominent role for MMP9 is implicated based on association of elevated levels in stroke patients with worsened outcomes and supported by studies demonstrating deterioration of the BBB by MMP9 due to degradation of vascular basement membranes (76). Moreover, MMP9 increases BBB permeability and degrades neuronal laminin resulting in neuronal apoptosis (76). Hypoxia sensitises brain endothelial cells to tPA-induced NFκB activation and MMP9 upregulation. Anti-inflammatory effects of APC include prevention of NFκB activation and nuclear translocation, thereby abrogating tPA-induced MMP9 expression (38). Other anti-inflammatory effects of APC inhibit expression of endothelial adhesion molecules that facilitate infiltration of the neurovascular unit by neutrophils, which are another source of MMP9 (33, 76). Thus, MMP9 is a major target for prevention of tPA-induced haemorrhagic transformation by APC.

Inside the neurovascular unit, APC is shown to protect murine and rat neurons, astrocytes and other brain cells from neuronal apoptosis induced by N-methyl-D-aspartate (NMDA), glutamate, and hypoxia (36, 37, 52, 56, 77, 78). These neuronal anti-apoptotic activities of APC require PAR1, PAR3 and EPCR and involve inhibition of nuclear localisation of apoptosis-inducing factor (AIF) and NFκB, inhibition of p53 induction, and inhibition of caspase 8 and 9 activation (36, 56). Transport of tPA across the BBB by low-density lipoprotein receptor related protein (LRP) results in neurotoxic effects on cells within the neurovascular unit (37, 75). APC’s anti-apoptotic activities inhibited augmentation of hypoxia- or NMDA-induced neuronal apoptosis by tPA that involved both mitochondria-mediated (caspase 9-dependent) intrinsic apoptotic pathways as well as extrinsic (caspase 8-dependent) apoptotic pathways (37). In summary, major neuroprotective effects of APC are centered on the triad of: (i) barrier protective effect on endothelial BBB cells, (ii) anti-inflammatory effects on multiple cells that prevent MMP9-mediated BBB breakdown, and (iii) anti-apoptotic effects on neurons and other cells inside the neurovascular unit (Figure 5) (30).

The window of opportunity for benefits from neuroprotective agents may be limited and generally coincides with the window of reperfusion benefit after stroke onset unless neuroprotective agents also have neuro-regenerative activities (13). In addition to APC neuroprotective effects, several lines of evidence suggest that APC promotes neuro-regeneration by inducing neovascularisation and neurogenesis (79, 80), stimulating production of neuronal progenitor cells (81), and promoting hippocampal metaplasticity associated with long term potentiation (82). These activities of APC may help explain its ability to improve neurological outcome when given beginning as late as 12 h to seven days after onset of ischaemia (79, 80). Thus, APC provides neuroprotective effects during the reperfusion phase, prevents haemorrhagic conversion and neurotoxic effects caused by tPA, and promotes vascular and neuronal repair in the postischaemic brain.

Figure 5: Multiple neuroprotective effects of APC on the neurovascular unit following ischaemic injury. APC neuroprotective effects and its ability to prevent tPA-induced neurotoxicity in the neurovascular unit include (i) protection of the BBB integrity, (ii) anti-inflammatory and anti-apoptotic effects on brain endothelial cells, and (iii) anti-apoptotic effects on neurons and other brain cells. This figure was modified from Zlokovic and Griffin, Trends Neuroscience 2011 (30).
3K3A-APC: preclinical and clinical development

The extensive studies on the effects of APC in the brain formed the impetus for translation of 3K3A-APC as adjuvant neuroprotective therapy to tPA in acute ischaemic stroke. Translation of promising preclinical neuroprotectants into stroke therapy has been proven difficult and unsuccessful thus far. The Stroke Therapy Academic Industry Roundtable (STAIR) formulated criteria to increase chances of success of drug development for stroke that were adapted to a STAIR quality scoring system (83). Development of human 3K3A-APC adheres to the STAIR recommendations and scores a perfect 10 for neuroprotective monotherapy and nine out of 10 for adjuvant therapy with tPA in ischaemic stroke (30, 58).

Some insights for APC-based therapies for stroke follow from experiences for recombinant wild-type APC (DrotAA; Xigris®) therapy for the treatment of adult severe sepsis that was FDA- and EAMA-approved and used for thousands of patients until it was withdrawn due to lack of efficacy in a second phase 3 trial conducted 10 years after the first very successful trial (84, 85). DrotAA therapy employed a 96 h-infusion of low-dose APC (24 µg/kg/h) based on the incorrect assumption that APC’s anticoagulant actions would protect against sepsis. Similar to stroke preclinical studies, data from in vivo studies using receptor-deficient mice and engineered activity-selective APC mutants imply that APC’s cytoprotective actions would protect against sepsis. 

Table 2: Differences in clinical trial designs for two APC therapies.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Clinical Trial</th>
<th>Pathway</th>
<th>Target</th>
<th>Activity</th>
<th>Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult severe sepsis</td>
<td>wt-APC</td>
<td>Anticoagulant</td>
<td>Factors Va &amp; VIIIa</td>
<td>Anticoagulant: 100 %</td>
<td>Low dose continuous 24 µg/kg/hr</td>
</tr>
<tr>
<td>Acute Ischemic Stroke</td>
<td>tPA + 3K3A-APC</td>
<td>Cytoprotective</td>
<td>PAR1 &amp; PAR3</td>
<td>Cytoprotective: 100 %</td>
<td>High dose bolus 5x/12 hr</td>
</tr>
</tbody>
</table>

Patients may have been exacerbated by both the wrong hypothesised mechanism of action and by its dosing regimen, which failed to deliver doses high enough to trigger cytoprotective cell signalling. Furthermore, the 96 h-infusion of low dose APC in sepsis was associated with an increased risk of serious bleeding (86).

3K3A-APC therapy for ischaemic stroke is fundamentally different from previous wt-APC therapy in human adult severe sepsis because 3K3A-APC’s mode of action targets APC’s cytoprotective activities and is based on different concepts than DrotAA therapy in sepsis that targeted anticoagulant activities (86).

3K3A-APC therapy for ischaemic stroke patients using 3K3A-APC following tPA standard-of-care therapy in clinical trials (phase 2). High-dose bolus dosing is
consistent with the therapeutic mechanisms of 3K3A-APC inducing PAR1 and PAR3-mediated cytoprotective cell signalling. Many successful in vivo preclinical studies for APC therapy for ischaemic stroke employed bolus dosing. Interestingly, one study using continuous infusion failed to demonstrate beneficial effects (87). Bolus dosing also limits APC-mediated, ongoing systemic proteolysis of FV and FVIII that may promote bleeding due to consumption of these clotting factors (88).

A successful phase 1 study showed that 3K3A-APC was well tolerated at multiple, very high doses (up to 540 μg/kg) every 12 h that gave peak plasma levels of ~4, 500 ng/ml (Figure 6), which is ~100-times higher than levels of circulating wt-APC (<50 ng/ml) achieved in the PROWESS or PROWESS-SHOCK trials with 96 h low dose infusions (84, 85, 89). The phase 1 clinical data for 3K3A-APC support the concept that cytoprotective-selective APC with reduced anticoagulant activity can be dosed significantly higher than wt-APC while causing minimal perturbations to clotting times. The safety, tolerability, and activity of 3K3A-APC, following the use of tissue plasminogen activator (tPA) in subjects with ischaemic stroke will remain to be determined and this will be the subject of a phase 2 clinical trial initiated this year. In this trial, 3K3A-APC will be administered after tPA as a 15 min infusion every 12 h for up to five infusions and at four increasing dose levels in approximately one hundred adult participants who will be followed for 90 days (http://www.neuronext.org/neuronext-please-annouce-funding-our-fourth-approved-trial-safety-evaluation-3k3a-apc-ischaemic).

**Summarisation**

Research on the cytoprotective cell signalling actions of the protein C system has made great progress in the last decade. Activity-selective APC mutants have firmly established important roles of APC’s cytoprotective activities for beneficial effects in models of inflammatory and ischaemic injury including ischaemic stroke. The integration of non-canonical PAR activation by APC and PAR-mediated biased signalling provides a paradigm shift in our understanding why and how different proteases display functional selectivity for cytoprotective vs proinflammatory effects. Collectively, these major advances and the extensive studies on the effects of APC in the brain resulted in the translation of 3K3A-APC to clinical trials for acute ischaemic stroke. In spite of the withdrawal of wt-APC therapy from the market in 2011, the 3K3A-APC phase 2 ischaemic stroke clinical trial provides a beacon of hope for therapies using APC variants in the future.

**Abbreviations**

Blood-brain-barrier (BBB), activated protein C (APC), protease activated receptor (PAR), endothelial protein C receptor (EPCR), tissue-type plasminogen activator (tPA), matrix metalloprotease (MMP), N-methyl-D-aspartate (NMDA), middle cerebral artery occlusion (MCAO).

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**Author contributions**

LO Mosnier, BV Zlokovic, and JH Griffin wrote the manuscript.

**Conflicts of interest**

LO Mosnier, JH Griffin, and BV Zlokovic are inventors for subject matters related to cytoprotective, neuroprotective APC variants. JH Griffin is a consultant for ZZBiotech LLC. BV Zlokovic is the scientific founder of ZZBiotech LLC, a biotechnology company with a focus to develop activated protein C and its functional mutants for stroke and other neurological disorders.

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Coagulation proteases and CVD

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