Activated Protein C Analog Protects From Ischemic Stroke and Extends the Therapeutic Window of Tissue-Type Plasminogen Activator in Aged Female Mice and Hypertensive Rats

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Background and Purpose—3K3A-activated protein C (APC) protects young, healthy male rodents after ischemic stroke. 3K3A-APC is currently under development as a neuroprotectant for acute ischemic stroke in humans. Stroke Therapy Academic Industry Roundtable recommends that after initial studies in young, healthy male animals, further studies should be performed in females, aged animals, and animals with comorbid conditions. Here, we studied the effects of delayed 3KA-APC therapy alone and with tissue-type plasminogen activator (tPA) in aged female mice and spontaneously hypertensive rats.

Methods—We used Stroke Therapy Academic Industry Roundtable recommendations for ensuring good scientific inquiry. Murine recombinant 3K3A-APC (0.2 mg/kg) alone or with recombinant tPA (10 mg/kg) was given intravenously 4 hours after transient middle cerebral artery occlusion in aged female mice and rats and after embolic stroke in spontaneously hypertensive rat. 3K3A-APC was additionally administered within 3 to 7 days after stroke. The neuropathological analysis and neurological scores, foot-fault, forelimb asymmetry, and adhesive removal tests were performed within 7 and 28 days of stroke.

Results—In all models, tPA alone had no effects on the infarct volume or behavior. 3K3A-APC alone or with tPA reduced the infarct volume 7 days after the middle cerebral artery occlusion in aged female mice and embolic stroke in spontaneously hypertensive rat by 62% to 66% and 50% to 53%, respectively, significantly improved \((P<0.05)\) behavior, and eliminated tPA-induced intracerebral microhemorrhages. In aged female mice, 3K3A-APC was protective within 4 weeks of stroke.

Conclusions—3K3A-APC protects from ischemic stroke and extends the therapeutic window of tPA in aged female mice and in spontaneously hypertensive rat with a comorbid condition. (Stroke. 2013;44:00-00.)

Key Words: neuroprotectant ◼ protein C ◼ rats, spontaneously hypertensive ◼ stroke

Activated protein C (APC) is a protease that exerts 2 major distinct activities: (1) anticoagulant activity that is mediated by limited proteolysis of coagulation factors Va and VIIIa with contributions by various cofactors and (2) cytoprotective direct effects on cells including antiapoptotic and anti-inflammatory activities, alterations in gene expression, and stabilization of endothelial barriers.1 In the central nervous system, APC and its cytoprotective analogs exert direct vasculoprotective, neuronal protective, anti-inflammatory, and proneurogenic activities in vitro and in vivo.2 Multiple studies have shown beneficial effects of APC and its cytoprotective analogs in rodent models of ischemic stroke,3-14 brain trauma,15,16 spinal cord injury,17-19 and chronic neurodegeneration including amyotrophic lateral sclerosis20 and multiple sclerosis.21

The proteolytic activation of protease-activated receptor (PAR) 1 by APC has a major role in APC’s protection of central nervous system vascular cells, neurons, and neuronal progenitor cells.2 Recent studies have shown that activation of PAR1 by APC involves a novel cleavage of the receptor’s N-terminal domain at Arg46, which reveals a novel cryptic intramolecular pharmacophore ending at residue Asn47 that causes APC’s biased cytoprotective signaling.22 In addition, endothelial protein C receptor23,24,25 and sphingosine-1-phosphate receptor-1,24,25 and PAR3,26 endothelial protein C receptor27,28 and sphingosine-1-phosphate receptor-114 contribute to APC’s protection of endothelial and blood–brain barrier integrity and neural cells, respectively.

Mutations of APC residues outside the APC proteolytic active site result in greatly reduced anticoagulant activity without altering the in vitro or in vivo cytoprotective effects of APC.9-14,29,30 For example, replacement of 3 lysine residues 191 to 193 by 3 alanine residues produces 3K3A-APC.
with >90% loss of anticoagulant activity but with preserved cytoprotective activity.29,30 Such engineered APC recombinant mutants are promising therapeutic biologics for stroke and neurological disorders because they provide APC analogs with significantly diminished risk of serious intracerebral bleeding, whereas the cytoprotective and pharmacological activities of APC within the neurovascular unit are fully preserved as is its transport across the blood-brain barrier.32

3K3A-APC is currently under development as a neuroprotectant for acute ischemic stroke in humans.33 Preclinical studies have shown that 3K3A-APC protects young, healthy male rodents after ischemic stroke and has advantages compared with the recombinant wild type (wt)-APC including reduced risk for bleeding particularly when treatments are administered at later time points after stroke.9–13 Stroke Therapy Academic Industry Roundtable (STAIR) criteria indicate that after initial studies in young, healthy male animals, further studies should be performed in female animals, aged animals, and animals with comorbid conditions.34,35 Therefore, here we studied the effects of 3K3A-APC alone and in combination with tissue-type plasminogen activator (tPA), the only approved therapy for acute ischemic stroke in humans.33 Preclinical studies in young, healthy male animals have shown that 3K3A-APC protects young, healthy male rodents after ischemic stroke and significantly diminished risk of serious intracerebral bleeding, whereas the cytoprotective and pharmacological activities of APC within the neurovascular unit are fully preserved as is its transport across the blood-brain barrier.32

Permanent Distal MCAo in Mice
Permanent distal MCAo was performed in aged female C57Bl6 mice using a modified technique as previously reported.13 Briefly, the mice were anesthetized intraperitoneally with 100 mg ketamine/10 mg xylazine per kg body weight. Under the surgical microscope, the left common carotid artery was isolated through a neck incision and ligated using a 5-0 silk. A skin incision was made between the right orbit and tragus. The zygomatic arch was removed and temporal muscle retracted laterally. The mandible was retracted downward. The MCA was visible through the temporal semitranslucent surface of the skull. Craniectomy was performed by drilling with a 0.9-mm round burr. The inner layer of the skull was removed with fine forceps. The dura was carefully opened and the M1 branch of the middle cerebral artery exposed and coagulated using a cauteter, producing permanent distal MCAo. The wound was sutured, and rectal temperature was maintained at 36.5°C to 37.0°C during surgery and for 2 hours after MCAo using a feedback-controlled heating system.

Mice were randomly assigned to 4 treatment groups that received vehicle, only 3K3A-APC, only tPA, and the combination of 3K3A-APC + tPA. Vehicle, 3K3A-APC alone (0.2 mg/kg, 50% bolus/50% 30-minute infusion), tPA alone (10 mg/kg, 10% as a bolus and 90% as a 30-minute infusion), and 3K3A-APC (0.2 mg/kg, infused as above) and tPA (10 mg/kg, infused as above) were administrated intravenously 4 hours after stroke. When tPA and 3K3A-APC were administered together, tPA was given via the femoral vein and 3K3A-APC via the tail vein. 3K3A-APC (0.2 mg/kg, IP) was administered additionally at 1, 3, 5, and 7 days after stroke. Foot-fault tests,5,11,12 forelimb asymmetry tests,5,11,12 and adhesive removal tests5,11,12,39 were performed at 0, 1, 7, 14, 21, and 28 days after the MCAo. Mice were euthanized 7 or 28 days after the MCAo for neuropathological analysis. The operators responsible for surgical procedures and outcome assessments were blinded and unaware of group allocation throughout the experiments.

Focal Embolic Stroke in SHRs
The MCAo in male SHRs was occluded by placement of an embolus at the origin of the MCA, as described.5,12,20 Briefly, an embolus was prepared from femoral arterial blood of a donor rat 24 hours before the procedure. The rats were anesthetized with 3% isoflurane, and the anesthesia was maintained with 1.0% to 1.5% isoflurane. Rectal temperature was maintained at 37.0 ± 0.5°C using a feedback-regulated heating pad system. The right common carotid artery, the right external carotid artery, and the internal carotid artery were isolated via a

Materials and Methods
Reagents
Murine 3K3A-APC (KKK192-194AAA) was prepared by ZZ Biotech using a stable cell line generated in Chinese hamster ovary cells.28 Note that residue numbering differs by one number for the triple Lys residue sequence in mouse versus human protein C. Briefly, the Chinese hamster ovary cells were grown in suspension in CD OptiCHO medium (Invitrogen, Carlsbad, CA) containing 2 mmol/L CaCl₂, 10 mg/mL vitamin K, and 2 mmol/L GlutaMAX (Invitrogen) in a 2-L Biowave bioreactor for production. A 4-step purification procedure was used: capturing protein C using a column containing FFQ resin (GE Health); purification of protein C using an Uno Q column (BioRad, Richmond, CA); activation with recombinant human thrombin (ZymoGenetics, Seattle, WA); and removal of thrombin using a Uno Q column. The purity of 3K3A-APC was determined by reduced SDS-PAGE/silver staining. There was no detectable thrombin in the purified APC preparations based on thrombin time clotting assays using purified fibrinogen.

Before using 3K3A-APC, its enzymatic activity was determined by amidolytic assay. In addition, activated partial thromboplastin time clotting assays using human factor V–deficient plasma containing 4% mouse plasma as a source of factor V were used to determine the anticoagulant activity of 3K3A-APC compared with wt-APC, as we previously described.6 Consistent with previous findings for human 3K3A-APC,7,30 the murine 3K3A mutations decreased anticoagulant activity by ≈80% but fully preserved cytoprotective activity.

A fresh aliquot of 3K3A-APC was used each time on a given day. Before using 3K3A-APC, its enzymatic activity was determined by amidolytic assay. In addition, activated partial thromboplastin time clotting assays using human factor V–deficient plasma containing 4% mouse plasma as a source of factor V were used to determine the anticoagulant activity of 3K3A-APC compared with wt-APC, as we previously described.6 Consistent with previous findings for human 3K3A-APC,7,30 the murine 3K3A mutations decreased anticoagulant activity by ≈80% but fully preserved cytoprotective activity. A fresh aliquot of 3K3A-APC was used each time on a given day of experiments. Human recombinant tPA (Alteplase) was purchased from Genentech (South San Francisco, CA).

Animals
All procedures were approved by the Institutional Animal Care and Use Committees at the University of Southern California (Zlokovic laboratory) and Cedars-Sinai Medical Center (Lyden laboratory) in compliance with the National Institutes of Health guidelines. Experiments in aged female mice and male SHRs were performed in the Zlokovic laboratory. Experiments in male Sprague–Dawley rats were performed in the Lyden laboratory. Aged female C57Bl6 mice (16 months old, 25–30 g) were purchased from the National Institute on Aging (Bethesda, MD). Male SHRs (9–10 weeks) were purchased from Charles River Laboratories (Wilmington, MA). Male Sprague–Dawley rats were purchased from Harlan Laboratories (San Diego, CA). All animals were randomized for all stroke studies and treatments. All experiments were blinded with respect to the operators responsible for surgical procedures and outcome assessments. Operators were blinded and unaware of group allocation throughout the experiments. For sample size calculations, see section Statistical Analysis.

Inclusion and Exclusion Criteria
Animals with an adequacy of middle cerebral artery occlusion (MCAo) as evidenced by ≥80% drop in the cerebral blood flow determined by Laser Doppler Flowmetry (Transonic System Inc) were included in the study.

Animals were excluded from the analysis when the following occurred: subarachnoid hemorrhage on postmortem analysis (2 SHRs), inadequacy of MCAo as evidenced by incomplete occlusion as described above (2 aged female mice and 3 SHRs), and death because of anesthesia or surgery that occurred within 4 hours of stroke induction (3 aged female mice and 2 SHRs).

3K3A-APC and tPA Doses
In all studies, we tested murine recombinant 3K3A-APC at a dose of 0.2 mg/kg that has been shown previously to exert an optimal protective effect in young male mice subjected to stroke.9,10 The dose of human recombinant tPA (10 mg/kg) was a standard dose typically used in rodents.4,6,12
midline incision. A modified E-50 catheter (0.3-mm outer diameter) filled with a single intact, fibrin-rich, homologous clot was gently inserted from the external carotid artery into the lumen of internal carotid artery, and the clot was positioned at the origin of the MCA.40 SHRs were randomly assigned to 4 treatment groups receiving vehicle, 3K3A-APC alone, tPA alone, and the combination of tPA+3K3A-APC. Vehicle, 3K3A-APC alone (0.2 mg/kg as a single bolus in 100 μL), tPA alone (10 mg/kg, 10% as a bolus and 90% as a 30-minute infusion), and the combination of 3K3A-APC (0.2 mg/kg administered as above) and tPA (10 mg/kg, infused as above) were administered intravenously 4 hours after stroke. 3K3A-APC (0.2 mg/kg) was additionally injected intravenously for 3 consecutive days. A modified neurological severity score, a composite of motor, sensory, reflex, and balance tests (no deficit, score 0; maximal deficit, score 18),5,12,41 was performed 1 and 7 days after stroke. Rats were euthanized 7 days after stroke for neuropathological analysis. The operators responsible for surgical procedures and outcome assessments were blinded and unaware of group allocation throughout the experiments.

Transient Proximal MCAo in Rats
Before performing extensive studies in the rat embolic stroke model (see above), transient MCAo in 3-month-old male Sprague–Dawley rats was performed in a limited number of animals to determine whether murine 3K3A-APC was effective in rats because there are well-known species-related differences as described previously for different murine and human APC preparations in different species.9,10 Briefly, rats weighing 290 to 310 g underwent transient MCAo surgery as described.32 Rats were divided into 2 groups receiving either saline or 3K3A-APC (0.2 mg/kg, 10-minute infusion) at 4 hours after stroke. Animals were anesthetized with 4% isoflurane mixed in oxygen and nitrous oxide (30:70). A midline neck incision was made exposing the left common carotid artery. The external carotid and pterygopalatine arteries were ligated with 4-0 silk suture and an incision was made in the wall of the external carotid artery close to the bifurcation point of the external and internal carotid arteries. A 4-0 heat-blunted nylon suture (Ethicon) was used for blocking the MCA and inserted and advanced 17.5 mm from the bifurcation point into the internal carotid arteries and kept in place for 2 hours. For saline and 3K3A-APC treatment, the jugular vein was isolated and a polyethylene 10 catheter inserted and secured with 6-0 silk ligatures. After the 2-hour occlusion duration, the nylon suture was removed from carotid artery to allow the perfusion of blood flow into the MCA. After the 2 hours of reperfusion, either saline or 3K3A-APC was infused into the jugular vein via a placed catheter using a syringe pump. At 0.2 mL/10 minutes. Neurological function was examined during reperfusion and 24 hours after onset of ischemia using a rodent neurological grading system.41 Animals were tested for forelimb withdrawal when suspended by tail, twisting of animal toward contralateral side, and circling behavior. For each abnormal finding, animals were given score of 1 point for a total possible score of 3. Animals were killed with an overdose of ketamine and xylazine, and then intracardially perfused with 250 mL of saline followed by 250 mL of 4% paraformaldehyde, 24 hours after the onset of ischemia. Brains were removed, fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose in phosphate buffer, and then sliced into 50-μm sections on a freezing microtome (Reichert-Jung). As in other studies, the operators responsible for surgical procedures and outcome assessments were blinded and unaware of group allocation throughout the experiments.

Physiological Measurements
Physiological parameters, including arterial blood pressure, blood gases, and pH, were monitored before surgery and at 1.0 hours after surgery, as we described.9,41 There were no difference in blood pressure, PaO2, PaCO2, or pH between the control groups and treatment groups (Wang et al, unpublished data, 2013).

Neuropathological Analysis
The injury volumes were measured on coronal sections using either cresyl-violet staining (mice) or hematoxylin and eosin staining (rats), as described.12,44,45 The infarct volume was calculated by subtracting the volume of intact area in the ipsilateral hemisphere from the whole volume of the contralateral hemisphere, as reported.5,4,40,41,44,46

Assessment of Cerebral Cortical Expansion
The aging female mice were perfusion fixed with paraformaldehyde 28 days after stroke and the brains were removed. Whole brain images were captured using a microscopic digital camera coupled to a dissection stereomicroscope (AxioCam, Zeiss). Images were analyzed by the National Institutes of Health ImageJ system (Bethesda, MD). The distance from midline to the edge of brain on the ischemic hemisphere was divided by the distance from midline to the lateral edge on the contralateral side to calculate the cortical width index as described.13,47,49

Neurodegeneration Analysis
In some studies, fluoro-Jade C was used to determine the MCAo-induced neurodegeneration in rats as described.49 Cellular injury of ischemic brain sections was imaged at low power using epifluorescence microscopy with a highly sensitive charge-coupled device camera (Apogee, Alta U32). In brief, for quantification of fluoro-Jade C positive neurons, National Institutes of Health ImageJ software was used. Operators blind to the treatment groups converted data to 8-bit, and for pseudo flat field; plugin was used to render uniform the fluorescence in all the sections. Using a nucleus counter particle analysis plugin, the number of stained neurons from each brain section was automatically counted.

Hemoglobin Assay
Hemoglobin levels were determined by a spectrophotometric assay using Drabkin reagent (Sigma).12,14

Measurement of Microscopic Hemorrhage
Microscopic hemorrhage area (mm2) was defined as the brain area which contained extravasated erythrocytes, as described.32 Briefly, 7 days after induction of ischemia, animals were euthanized and perfused through the heart with 10 U/mL heparin in 0.9% saline, followed by 4% paraformaldehyde. The brain was rapidly removed and embedded in optimal cutting temperature compound. Eight unstained coronal sections of the brain (20 μm in thickness and 1 mm apart) were collected and imaged using a microscopic digital camera system coupled to a dissection stereomicroscope (AxioCam, Zeiss). Images were analyzed by the National Institutes of Health ImageJ system (Bethesda, MD).

Statistics
Data are presented as mean±SD. One-way ANOVA followed by Tukey post hoc test was used to determine statistically significant differences. P<0.05 was considered statistically significant.

Sample sizes were calculated using NQUERY assuming a 2-sided α-level of 0.05, 80% power, and homogeneous variances for the 2 samples to be compared, with the mean and common SD predicted from pilot data and previous studies.5,9,10,12,13,40,42,43

Results
The goal of our first set of experiments was to determine whether administration of murine recombinant 3K3A-APC (0.2 mg/kg) alone or in combination with tPA (10 mg/kg) at 4 hours after transient distal MCAo protects aged female mice within 7 days of stroke. 3K3A-APC (0.2 mg/kg) was additionally administered 1, 3, 5, and 7 days after stroke. Our data show that 3K3A-APC alone reduced the infarct and edema volumes by 62% and 58%, respectively, whereas tPA alone did not have any significant effect on ischemic injury compared with vehicle...
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(Figure 1A–1C). Moreover, tPA alone increased the risk for intracerebral bleeding as indicated by significantly increased hemoglobin levels in the ischemic hemisphere (Figure 1D). A combined 3K3A-APC and tPA treatment resulted in significant reductions in the infarct and edema volumes by 66% and 62% compared with vehicle that was similar to the results obtained with 3K3A-APC alone (Figure 1A–1C). 3K3A-APC eliminated hemoglobin accumulation in the ischemic hemisphere seen with tPA alone (Figure 1D), consistent with a significant vasculoprotective effects of 3K3A-APC and wt-APC observed previously in young male ischemic mice.5,6,12 As expected based on neuropathological findings, 3K3A-APC improved behavioral outcome in aged female mice. Significant improvements were found on foot-fault tests and forelimb asymmetry tests at 1 and 7 days after stroke (Figure 2A and 2B), suggesting the respective improvements in locomotor assessment and sensorimotor activity.2

Next, we investigated whether 3K3A-APC beneficial effects seen within 7 days of stroke remain significant during longer periods of time such as within 4 weeks of stroke. 3K3A-APC given after transient distal MCAo as above improved significantly the cortical width index by 45% compared with vehicle (Figure 3A and 3B). Cortical width index is used commonly as a measure of cerebral cortical expansion to determine the effects of posts ischemic treatments after longer periods of time after stroke in rodents.13,47,48 tPA alone did not have an effect on the cortical width index in contrast to the combined 3K3A-APC and tPA therapy that increased the cortical width index by 48% (Figure 3A and 3B). Consistent with these findings, 3K3A-APC alone and in combination with tPA improved significantly locomotor assessment and sensorimotor activity as determined by foot-fault and adhesive removal test, respectively, at 1, 7, 14, 21, and 28 days after stroke (Figure 3C and 3D). As expected based on neuropathological findings (Figure 1A–1C), tPA alone did not exert any beneficial neurological effects within the 4 weeks of follow-up when it was administered 4 hours after stroke.

Next, we studied whether murine 3K3A-APC at the highest protective dose used in mice6,10 can exert neuroprotection in rats after MCAo. Murine 3K3A-APC has never been tested in rats before. Given the reported species differences in the efficacy of different human and murine APC preparations in different species,10 we thought that before testing murine 3K3A-APC in SHR embolic model we should find out first whether 0.2 mg/kg of murine 3K3A-APC provides any benefit to rats after stroke. Our data indicate that murine 3K3A-APC exerts strong neuroprotection 24 hours after 2-hour transient proximal MCAo in rats as evidenced by a significant 35% improvement in the Bederson neurological score6 and 42% reduction in the number of degenerating Fluoro-Jade positive neurons (Figure 4A and 4B).

Based on the encouraging data obtained with murine 3K3A-APC in rats after MCAo (Figure 4), we next studied the effects of murine recombinant 3K3A-APC (0.2 mg/kg) alone or in combination with tPA (10 mg/kg) given at 4 hours after embolic stroke in SHRs. 3K3A-APC (0.2 mg/kg) was additionally injected intravenously for 3 consecutive days. Murine 3K3A-APC alone reduced by a remarkable 53% the infarct volume within 7 days of embolic stroke in SHRs, whereas tPA alone did not have an effect on the infarct volume (Figure 5A and 5B). A combined 3K3A-APC and tPA treatment reduced

Figure 1. Effects of murine 3K3A-activated protein C (APC) and a combined 3K3A-APC and tissue-type plasminogen activator (tPA) treatment on neuropathological outcome in aged female mice within 7 days of distal permanent middle cerebral artery occlusion (MCAo). 3K3A-APC (0.2 mg/kg) and tPA (10 mg/kg) were administered intravenously for 3 consecutive days. Murine 3K3A-APC has never been tested in different species,10 we thought that before testing murine 3K3A-APC in SHR embolic model we should find out first whether 0.2 mg/kg of murine 3K3A-APC provides any benefit to rats after stroke. Our data indicate that murine 3K3A-APC exerts strong neuroprotection 24 hours after 2-hour transient proximal MCAo in rats as evidenced by a significant 35% improvement in the Bederson neurological score6 and 42% reduction in the number of degenerating Fluoro-Jade positive neurons (Figure 4A and 4B).

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Figure 2. Effects of murine 3K3A-activated protein C (APC) on functional outcome in aged female mice with and without tissue-type plasminogen activator (tPA) treatment after stroke in rodents.13,47,48 tPA alone did not have an effect on the cortical width index in contrast to the combined 3K3A-APC and tPA therapy that increased the cortical width index by 48% (Figure 3A and 3B). Consistent with these findings, 3K3A-APC alone and in combination with tPA improved significantly locomotor assessment and sensorimotor activity as determined by foot-fault and adhesive removal test, respectively, at 1, 7, 14, 21, and 28 days after stroke (Figure 3C and 3D). As expected based on neuropathological findings (Figure 1A–1C), tPA alone did not exert any beneficial neurological effects within the 4 weeks of follow-up when it was administered 4 hours after stroke.
the infarction volume by 55% compared with vehicle; a similar reduction was observed compared with the tPA alone treatment group (Figure 5A–5C). Similar to the data for aged female mice, tPA increased the area of microscopic hemorrhage by 3.5-fold compared with vehicle or 3K3A-APC alone (Figure 5C). 3K3A-APC alone significantly reduced tPA's risk for bleeding as shown by normalization of the microscopic hemorrhage area toward values found in vehicle-treated controls (Figure 5C). Consistent with neuropathological data, both 3K3A-APC alone and 3K3A-APC combined with tPA improved by >50% modified neurological severity score scores at 1 and 7 days after embolic stroke compared with either vehicle or tPA alone (Figure 5D). Consistent with the reported species differences in the efficacy of murine and human APC preparations in rodents,7 murine 3K3A-APC used in the present study was more potent in protecting rats from embolic stroke than human 3K3A-APC in a previous study.12

**Discussion**

Consistent with previous studies in healthy young male rodents,9–11 the present study shows that murine 3K3A-APC is protective in aged female mice, young male rats, and male SHRs when administered 4 hours after stroke. In contrast, tPA alone did not show beneficial effects in the present models of stroke consistent with some previous studies demonstrating that tPA is ineffective when given relatively late to rodents after the MCAo embolism12,53 or transient MCAo.4,54,55 Nevertheless, thrombolytic therapy for acute ischemic stroke with tPA has clear benefits if administered early within a narrow therapeutic window as reviewed elsewhere.56–58 The present study also shows that 3K3A-APC extends the therapeutic window of tPA after transient MCAo in aged female mice and the MCAo embolism in SHRs confirming previous findings that wt-APC4,6 and 3K3A-APC12 widen the therapeutic window of tPA for ischemic stroke in young healthy male rodents. We also show that 3K3A-APC alone or in combination with tPA exerts beneficial effects on neuropathological and behavioral outcomes in aged female mice during a longer period of 4 weeks.

According to the recommended STAIR criteria34,35 and a modified scoring STAIR system with a focus on the scope of testing across experimental models from 0 to 10 (with 10 being the highest beneficial score),56 the STAIR quality score for wt-APC is 8.2 For comparison, recombinant tPA has a score of 9.31 With previous studies8–13 and the current study the STAIR quality score for 3K3A-APC alone and a combined 3K3A-APC and tPA therapy is 10 and 9, respectively, as illustrated in the Table. The criteria listed in the Table largely reflect the STAIR recommendations8–13 but are also modified as reported by O’Collins et al.56 For example, testing in primates was not included as a criterion because the superior validity of primate models has not been well established and has not been included in STAIR analysis of other stroke drugs.56 It is of note, however, that APC’s beneficial effects in

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**Figure 3.** Effects of murine 3K3A-activated protein C (APC) and a combined 3K3A-APC and tissue-type plasminogen activator (tPA) treatment in aged female mice within 28 days of distal permanent middle cerebral artery occlusion (MCAo). 3K3A-APC (0.2 mg/kg) and tPA (10 mg/kg) were administered intravenously 4 hours after the MCAo. 3K3A-APC (0.2 mg/kg, IP) was additionally given at 1, 3, and 5 days after the MCAo. 3K3A-APC (0.2 mg/kg) was additionally given at 1 and 7 days after embolic stroke compared with either vehicle or tPA alone (Figure 5C). 3K3A-APC alone significantly reduced tPA's risk for bleeding as shown by normalization of the microscopic hemorrhage area toward values found in vehicle-treated controls. The average cortical width index calculated as the ratio of a divided by b. The average cortical width index determined in different treatment groups 28 days after the MCAo. A, Cortical cavitation (top) and measurements of the cortical width index (bottom) using National Institutes of Health ImageJ software in mice under different treatments were performed 28 days after stroke: a, maximum width from midpoint to the edge of infarcted hemisphere; b, maximum width from midpoint to the edge of noninfarcted hemisphere; cortical width index was calculated as the ratio of a divided by b. B, The average cortical width index determined in different treatment groups 28 days after the MCAo. C, Foot-fault test and D adhesive removal tests were performed at day 0, 1, 7, 14, 21, and 28 after stroke. All values are mean±SD, n=5 mice per group. *P<0.01, 3K3A-APC alone vs vehicle or tPA alone; **P<0.01, tPA+3K3A-APC vs vehicle or tPA alone; ns, nonsignificant tPA alone vs vehicle. The average cortical width index determined in different treatment groups 28 days after the MCAo. A, Cortical cavitation (top) and measurements of the cortical width index (bottom) using National Institutes of Health ImageJ software in mice under different treatments were performed 28 days after stroke: a, maximum width from midpoint to the edge of infarcted hemisphere; b, maximum width from midpoint to the edge of noninfarcted hemisphere; cortical width index was calculated as the ratio of a divided by b. B, The average cortical width index determined in different treatment groups 28 days after the MCAo. C, Foot-fault test and D adhesive removal tests were performed at day 0, 1, 7, 14, 21, and 28 after stroke. All values are mean±SD, n=5 mice per group. *P<0.01, 3K3A-APC alone vs vehicle or tPA alone; **P<0.01, tPA+3K3A-APC vs vehicle or tPA alone; ns, nonsignificant tPA alone vs vehicle.

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**Figure 4.** Effects of murine 3K3A-activated protein C (APC) treatment alone on neurological functions and neurodegeneration after middle cerebral artery occlusion (MCAo) in rats. Rats administered intravenously with 3K3A-APC (0.2 mg/kg) 4 hours after MCAo were compared with saline-treated animals based on Bederson scale. A, Neurological scores 24 hours after MCAo show significant recovery of neurological functions in 3K3A-APC–treated animals. B, Fluoro-Jade C, a marker for labeling degenerated neurons, was used to determine the neuroprotective effect of 3K3A-APC. Single dose of 3K3A-APC administered 4 hours after MCAo resulted in significantly reduced number of degenerated neurons in comparison with control (saline treated) animals. The degenerated neurons were counted using National Institutes of Health ImageJ software. All values are mean±SEM, n=8 rats per group. *P<0.01, 3K3A-APC vs saline 24 hours after MCAo; ***P<0.001, 3K3A-APC vs saline.
nonhuman primates have been shown in models of sepsis and arterial thrombosis, as reviewed elsewhere.1

By analyzing methodological quality and efficacy of 1026 stroke drugs tested in >8500 experiments in 3500 publications, O'Collins et al56 determined that only 5 drugs met the STAIR criteria for drug development for stroke. The present study was performed according to revised STAIR recommendations35 for ensuring good scientific inquiry including the following: inclusion and exclusion criteria, the method of allocation, randomization, blinded assessment of outcome, and sample size calculations as described in the Material and Methods. We also reported in the disclosures potential conflicts of interest and study funding.

Notably, investigators have learned for years that adherence to the STAIR criteria does not guarantee success in clinical applications in humans. For example, failure of the

Table.  STAIR Quality for 3K3A-APC Alone Therapy and for the 3K3A-APC and tPA Combination Therapy Using the Experimental Stroke Scale Modified by O'Collins et al56

<table>
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<tr>
<th>STAIR Criterion</th>
<th>Description</th>
<th>3K3A-APC Score</th>
<th>Refs</th>
<th>3K3A-APC/tPA Score</th>
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<td>12, present</td>
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<td>Focal model in ≥2 species</td>
<td>Yes</td>
<td>9–13</td>
<td>Yes</td>
<td>12, present</td>
</tr>
<tr>
<td>Health</td>
<td>Focal model in old or diseased animals</td>
<td>No</td>
<td>Present</td>
<td>Yes</td>
<td>Present</td>
</tr>
<tr>
<td>Sex</td>
<td>Focal model in males and females</td>
<td>Yes</td>
<td>Present</td>
<td>Yes</td>
<td>Present</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>Tested in temporary and permanent models</td>
<td>Yes</td>
<td>9–13</td>
<td>Yes</td>
<td>12</td>
</tr>
<tr>
<td>Time window</td>
<td>Administered ≥1 h after occlusion</td>
<td>Yes</td>
<td>9–13</td>
<td>Yes</td>
<td>12, present</td>
</tr>
<tr>
<td>Doses</td>
<td>Administered using ≥2 doses</td>
<td>Yes</td>
<td>10,11</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Route</td>
<td>Using a feasible model of delivery</td>
<td>Yes</td>
<td>9–13</td>
<td>Yes</td>
<td>12</td>
</tr>
<tr>
<td>End point</td>
<td>Both behavioral and histological outcomes</td>
<td>Yes</td>
<td>9–13</td>
<td>Yes</td>
<td>12</td>
</tr>
<tr>
<td>Long term</td>
<td>Outcome measured at 4 wk</td>
<td>Yes</td>
<td>Present</td>
<td>Yes</td>
<td>Present</td>
</tr>
</tbody>
</table>

STAIR criteria are not given in the order of priority. Present data are used from the present study. APC indicates activated protein C; STAIR, Stroke Therapy Academic Industry Roundtable; and tPA, tissue-type plasminogen activator.
Stroke-Acute Ischemic NXY Treatment (SAINT) II trial evaluating the free radical scavenger NXY-059 in acute ischemic stroke has prompted discussions about the quality of preclinical and clinical stroke studies. Although most stroke investigators would agree that there is no evidence to suggest a biological barrier to translating stroke research from animals to humans, the quality of preclinical research has been identified often as a potential problem. In addition, it has been argued that the design of clinical trials may have often underestimated the sample size needed to show an effect based on conventional outcome scales. In addition, computed tomography and MRI of infarct volumes frequently fail to show the correlation with clinical outcomes, making these imaging biomarkers unreliable as validated end points sufficient to grant approval of a neuroprotectant drug by regulatory agencies.

As a solution to these problems, it has been suggested that a candidate drug even before testing in animal models should have a clearly defined molecular mechanism of action, a valid molecular target, an acceptable toxicity profile, appropriate pharmacokinetics and pharmacodynamics, and a demonstrated ability to cross the blood-brain barrier. Interestingly, most of these criteria have been satisfied in case of APC biologics such as 3K3A-APC. The cellular and molecular mechanisms of cytoprotective actions of APC and 3K3A-APC in multiple models of peripheral organ injury (eg, heart, kidney, liver, and lung) and of central nervous system acute and chronic injury have been shown by multiple independent laboratories as reviewed elsewhere. For example, in regards to central nervous system, it has been shown that PAR1 is a key receptor mediating beneficial effects of APC and 3K3A-APC in brain endothelium, neurons, and microglia, and that activation of PAR1 by APC or 3K3A-APC inhibits the intrinsic, caspase-9 and the extrinsic, caspase-8 apoptotic pathways, enhances the integrity of endothelial barrier via Racl-dependent signaling, exerts anti-inflammatory effects, and leads to beneficial alterations in gene expression profiles. It has been also shown that APC and 3K3A-APC cross the blood-brain barrier.

In summary, preclinical studies including the present study support the development of APC biologics and 3K3A-APC as a therapy for stroke, administered either alone or in combination with tPA reperfusion treatment. Importantly, human recombinant 3K3A-APC has been manufactured as a neuroprotectant for ischemic stroke in humans, and its pharmacokinetics and safety profile have been reported in primates and rodents. The phase I safety trial in humans and pharmacokinetic studies in human volunteers have also been completed successfully, and the results will be reported in the near future (Lyden et al, unpublished data). Therefore, 3K3A-APC alone or in combination with tPA is well poised to move forward to the next stage of phase 2 studies in patients with stroke.

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Disclosures
Dr Zlokovic is the scientific founder of ZZ Biotech, a biotechnology company with a focus to develop activated protein C and its functional mutants for stroke and other neurological disorders. Dr Griffin is a member of the Scientific Advisory Board of ZZ Biotech LLC. Dr Lyden is consultant for ZZ Biotech LLC. The other authors report no conflicts.

References


Activated Protein C Analog Protects From Ischemic Stroke and Extends the Therapeutic Window of Tissue-Type Plasminogen Activator in Aged Female Mice and Hypertensive Rats

Yaoming Wang, Zhen Zhao, Nienwen Chow, Padmesh S. Rajput, John H. Griffin, Patrick D. Lyden and Berislav V. Zlokovic

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