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An activated protein C analog protects from ischemic stroke and extends the therapeutic window of tPA in aged female mice and hypertensive rats

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Abstract

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 (STAIR) 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 plasminogen activator (tPA) in aged female mice and spontaneously hypertensive rats (SHR).

Methods—We used STAIR 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 (MCAo) in aged female mice and rats, and after embolic stroke in SHR. 3K3A-APC was additionally administered within 3–7 days after stroke. The neuropathological analysis and neurological scores, foot-fault, forelimb asymmetry and/or 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 MCAo in aged female mice and embolic stroke in SHR by 62–66% and 50–53%, respectively, improved significantly ($p<0.05$) 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 SHR with a comorbid condition.

Keywords

ischemic stroke; proteases; neuroprotection; old female mice; hypertensive rats

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Disclosures

B.V.Z. is the scientific founder of ZZ Biotech, a biotechnology company with a focus to develop APC and its functional mutants for stroke and other neurological disorders. J.H.G. is a member of the Scientific Advisory Board of ZZ Biotech LLC. P.L. is consultant for ZZ Biotech LLC. Others do not have disclosures for any conflicts.

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Introduction

Activated protein C (APC) is a protease that exerts two major distinct activities: i) anticoagulant activity that is mediated by limited proteolysis of coagulation factors Va and VIIIa with contributions by various cofactors, and ii) cytoprotective direct effects on cells including anti-apoptotic and anti-inflammatory activities, alterations in gene expression, and stabilization of endothelial barriers.[1] In the central nervous system (CNS), APC and its cytoprotective analogs exert direct vasculoprotective, neuronal protective, anti-inflammatory and pro-neurogenic 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/or chronic neurodegeneration including amyotrophic lateral sclerosis[20] and multiple sclerosis.[21]

The proteolytic activation of protease activated receptor-1 (PAR1) by APC has a major role in APC's protection of CNS 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 receptor (EPCR)[5,6,23–25] and sphingosine-1-phosphate receptor-1 ($S1P_1$),24,25 and PAR3,[26] EPCR[27,28] and $S1P_1$14 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.[9–14,29–31] For example, replacement of 3 lysine residues 191–193 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 pharmacologic activities of APC within the neurovascular unit are fully preserved[2], as is its transport across the BBB.[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 over 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 3KA-APC alone and in combination with tissue plasminogen activator (tPA), the only approved therapy for ischemic stroke,[36–38] in aged female mice and spontaneously hypertensive rats (SHR).

Materials and Methods

Reagents

Murine 3K3A-APC (KKK192–194AAA) was prepared by ZZ Biotech using a stable cell line generated in Chinese hamster ovary (CHO) cells.[9] Note that residue numbering differs by one number for the triple Lys residue sequence in mouse vs human protein C. Briefly, the CHO cells were grown in suspension in CD OptiCHO medium (Invitrogen, Carlsbad, CA) containing 2 mM $CaCl_2$, 10 μg/ml vitamin K and 2 mM GlutaMAX (Invitrogen) in a 2 L Biowave bioreactor for production. A four-step purification procedure was used: capturing PC using a column containing FFQ resin (GE Health); purification of PC using an Uno Q

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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 (aPTT) 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 to wt-APC, as we previously described.[9] Consistent with previous findings for human 3K3A-APC,[29,30] the murine 3K3A mutations decreased anticoagulant activity by approximately 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^{TM}) 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 SHR 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 SHR (9–10 weeks of age) 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 (LDF, Transonic System Inc.) were included in the study.

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

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]

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Permanent Distal MCAo in mice

Permanent distal MCAo was performed in aged female C57Bl6 mice using a modified technique as previously reported.[11] 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 cauterizer, producing permanent distal MCAo. The wound was sutured, and rectal temperature was maintained at 36.5 – 37.0°C during surgery and for 2 hours after MCAo using a feedback-controlled heating system.

Mice were randomly assigned to four treatment groups that received vehicle, only 3K3A-APC, only tPA, or the combination of 3K3A-APC + tPA. Vehicle, 3K3A-APC alone (0.2 mg/kg, 50% bolus/50% 30 min infusion), tPA alone (10 mg/kg, 10% as a bolus and 90% as a 30 min 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, intraperitoneally) was additionally administered at 1, 3, 5, and 7 days after stroke. Foot-fault tests,[5,11,12] forelimb asymmetry tests[5,11,12] and/or adhesive removal tests[5,11,12,39] 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 SHR

The MCAo in male SHR was occluded by placement of an embolus at the origin of the MCA, as described.[5,12,40] 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°C±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 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] SHR were randomly assigned to four treatment groups receiving vehicle, 3K3A-APC alone, tPA alone or 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 min infusion), or 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.

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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 290–310 g underwent transient MCAo surgery as described.[42] Rats were divided in two groups receiving either saline or 3K3A-APC (0.2 mg/kg 10 min 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. 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 PE 10 catheter inserted and secured with 6-0 silk ligatures. After the 2 hours occlusion duration, the nylon suture was removed from carotid artery to allow the reperfusion of blood flow into the MCA. After the 2 hours of reperfusion, either saline or 3K3A-APC were infused into the jugular vein via a placed catheter using a syringe pump at 0.2 ml over 10 min. Neurological function was examined during reperfusion and 24 h after onset of ischemia using a rodent neurological grading system.[43] Animals were tested for forelimb withdrawal when suspended by tail, twisting of animal towards contra-lateral 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 h 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 h after surgery, as we described.[9,11] There were no difference in blood pressure, pO2, pCO2 or pH between the control groups and treatment groups (data not shown).

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.[3–6,40,41,44,46]

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 system coupled to a dissecting stereomicroscope (AxioCam, Zeiss). Images were analyzed by the NIH Image J system (Bethesda, MD, USA). 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.[13,47,48]

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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 CCD camera (Apogee, Alta U32). In brief, for quantification of fluoro-Jade C positive neurons, NIH 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. By 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).[6,12,50]

Measurement of Microscopic Hemorrhage

Microscopic hemorrhage area (mm^2) was defined as the brain area which contained extravasated erythrocytes, as described.[51] 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% PFA. The brain was rapidly removed and embedded in OCT. 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 dissecting stereomicroscope (AxioCam, Zeiss). Images were analyzed by the NIH Image J system (Bethesda, MD, USA).

Statistics

Data are presented as mean ± standard deviation. One-way analysis of variance 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 two-sided alpha-level of 0.05, 80% power, and homogeneous variances for the 2 samples to be compared, with the means and common standard deviation predicted from pilot data and previous studies.[5,9,10,12,13,40,42,52]

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 to vehicle (Fig. 1A–C). Moreover, tPA alone increased the risk for intracerebral bleeding as indicated by significantly increased hemoglobin levels in the ischemic hemisphere (Fig. 1D). A combined 3K3A-APC and tPA treatment resulted in significant reductions in the infarct and edema volumes by 66% and 62% compared to vehicle, that was similar to the results obtained with 3K3A-APC alone (Fig. 1A–C). 3K3A-APC eliminated hemoglobin accumulation in the ischemic hemisphere seen with tPA alone (Fig. 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

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after stroke (Fig. 2A–B) suggesting the respective improvements in locomotor assessment and sensorimotor activity.[12]

Next, we asked whether 3K3A-APC beneficial effects seen within 7 days of stroke remain significant over 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 to vehicle (Fig. 3A–B). Cortical width index is commonly used as a measure of cerebral cortical expansion to determine the effects of post-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% (Fig. 3A–B). 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 (Fig. 3C–D). As expected based on neuropathological findings (Fig. 1A–C), 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 mice[9,10] 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 felt that prior to 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 hours transient proximal MCAo in rats as evidenced by a significant 35% improvement in the Bederson's neurological score[43] and 42% reduction in the number of degenerating Fluoro-Jade positive neurons (Fig. 4A–B).

Based on the encouraging data obtained with murine 3K3A-APC in rats post MCAo (Fig. 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 SHR. 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 SHR, whereas tPA alone did not have an effect on the infarct volume (Fig. 5A–B). A combined 3K3A-APC and tPA treatment reduced the infarction volume by 55% compared to vehicle; a similar reduction was observed compared to the tPA alone treatment group (Fig. 5A–C). Similar to the data for aged female mice, tPA increased the area of microscopic hemorrhage by 3.5-fold compared to vehicle or 3K3A-APC alone (Fig. 5C). 3K3A-APC alone significantly reduced tPA's risk for bleeding as shown by normalization of the microscopic hemorrhage area towards values found in vehicle-treated controls (Fig. 5C). Consistent with neuropathological data, both 3K3A-APC alone and 3K3A-APC combined with tPA improved by approximately 50% mNSS scores at 1 and 7 days after embolic stroke compared to either vehicle or tPA alone (Fig. 5D). Consistent with the reported species differences in the efficacy of murine and human APC preparations in rodents[10], 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[9–13], the present study shows that murine 3K3A-APC is protective in aged female mice, young male rats and male SHR 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 embolism,[12,53]

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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[36–38]. 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 SHR confirming previous findings that wt-APC[4,6] and 3K3A-APC[12] 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 over a longer period of 4 weeks.

According to the recommended STAIR criteria[34,35] 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[53]. With previous studies[9–13] 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 Table 1. The criteria listed in Table 1 largely reflect the STAIR recommendations[34,35] 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 nonhuman primates have been shown in models of sepsis and arterial thrombosis, as reviewed elsewhere.[1]

By analyzing methodological quality and efficacy of 1,026 stroke drugs tested in over 8,500 experiments in 3,500 publications, O'Collins et al. determined that only 5 drugs met the STAIR criteria for drug development for stroke[56]. The present study was carried out according to revised STAIR recommendations [35] 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 disclosures potential conflicts of interest and study funding.

Notably, investigators have learned over years that adherence to the STAIR criteria does not guarantee success in clinical applications in humans. For example, failure of the Stroke-Acute Ischemic NXY Treatment (SAINT) II trial evaluating the free radical scavenger NXY-059 in acute ischemic stroke[57] has prompted discussions about the quality of preclinical and clinical stroke studies[58]. 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[59]. In addition, computed tomography and magnetic resonance imaging of infarct volumes frequently fail to show the correlation with clinical outcomes[60–62] 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 BBB.[34,35,63] 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/or 3K3A-APC in multiple models of peripheral organ injury (e.g., heart, kidney, liver, lung) and of CNS acute and chronic injury have been shown by multiple independent laboratories as reviewed

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elsewhere[1,2]. For example, in regards to CNS 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 Rac1-dependent signaling, exerts anti-inflammatory effects and leads to beneficial alterations in gene expression profiles.[2] It has been also shown that APC and 3K3A-APC cross the BBB.[32]

In summary, preclinical studies including the present study support 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.[33] The Phase 1 safety trial in humans and pharmacokinetic studies in human volunteers have also been successfully completed, and the results will be reported in the near future (Lyden et al., unpublished observations). Therefore, 3K3A-APC alone or in combination with tPA is well poised to move forward to the next stage of Phase 2 studies in stroke patients.

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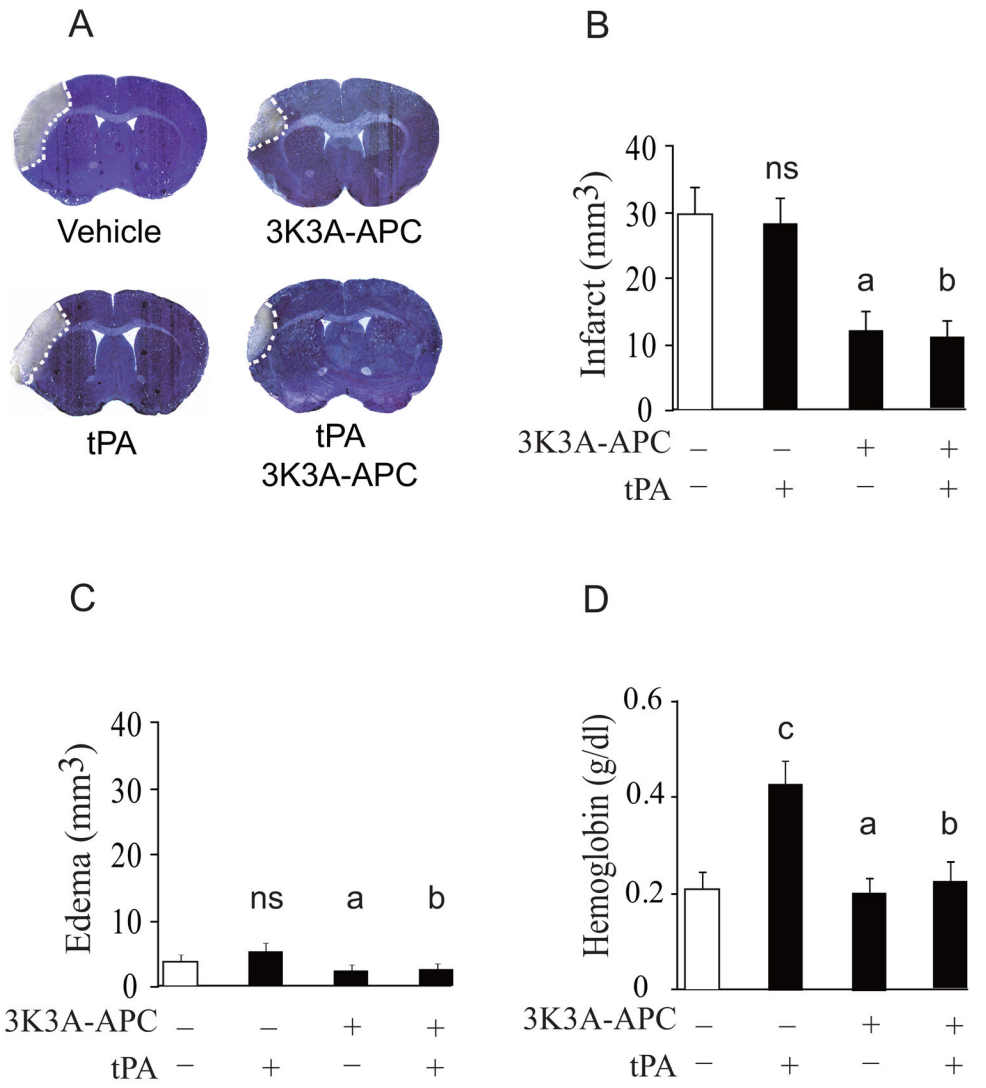

Figure 1. Effects of murine 3K3A-APC and a combined 3K3A-APC and tPA treatment on neuropathological outcome in aged female mice within 7 days of distal permanent MCAo

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, intraperitoneally) was additionally given at 1, 3, 5, and 7 days after the MCAo. (**A**) Cresyl-violet staining of brain coronal sections at the level of optic chiasm of mice under different treatments was performed 7 days after stroke. (**B**) Infarct volume, (**C**) edema and (**D**) hemoglobin levels in the ischemic hemisphere were determined 7 days after the MCAo. All values are mean ± SD, n = 5 mice per group. In B-D, $^{a}P < 0.01$, 3K3A-APC alone vs. vehicle or tPA alone; $^{b}P < 0.01$, tPA + 3K3A-APC vs. vehicle or tPA alone; In B-C, ns, non-significant tPA alone vs. vehicle; In D, $^{c}P < 0.01$, tPA alone vs. vehicle.

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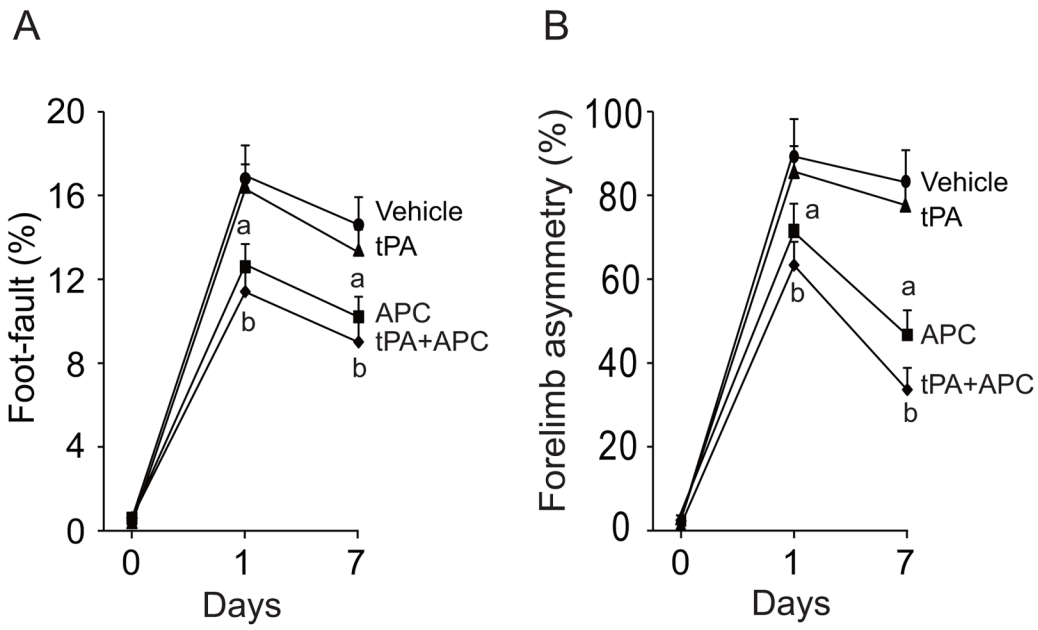

Figure 2. Effects of murine 3K3A-APC on functional outcome in aged female mice with and without tPA within 7 days of distal permanent MCAo

Animals were treated with 3K3A-APC (0.2 mg/kg) and tPA (10 mg/kg) intravenously 4 hours after the MCAo as described in Figure 1. 3K3A-APC (0.2 mg/kg, intraperitoneally) was additionally given at 1, 3, 5, and 7 days after the MCAo. (**A**) Foot-fault and (**B**) forelimb asymmetry tests were performed 1, 3 and 7 days after the MCAo. Animals were sacrificed after 7 days for neuropathological analysis shown in Figure 1. All values are mean ± SD, n = 5 mice per group. [a]$P < 0.01$, 3K3A-APC alone vs. vehicle or tPA alone; [b]$P < 0.01$, tPA + 3K3A-APC vs. vehicle or tPA alone.

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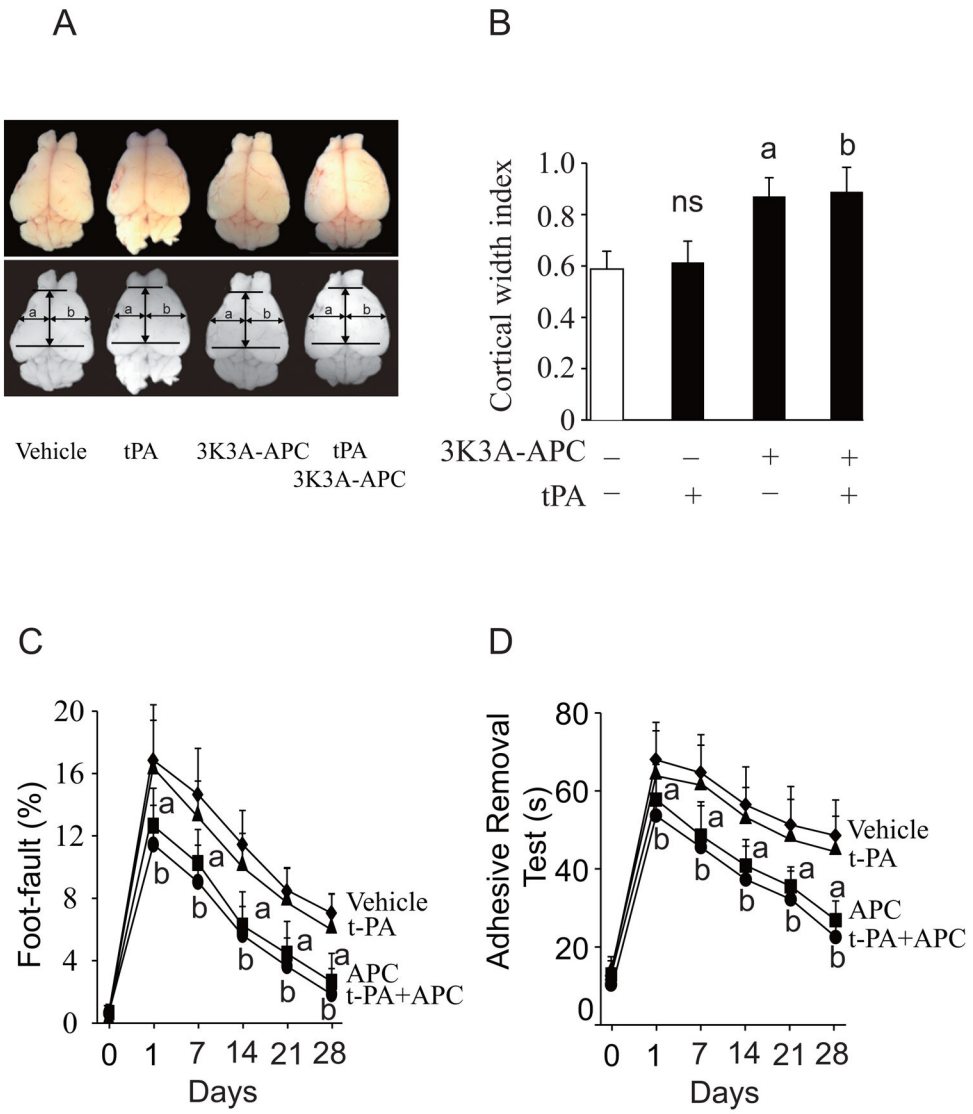

Figure 3. Effects of murine 3K3A-APC and a combined 3K3A-APC and tPA treatment in aged female mice within 28 days of distal permanent MCAo

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, intraperitoneally) was additionally given at 1, 3, 5, and 7 days after the MCAo. (**A**) Cortical cavitation (upper panels) and measurements of the cortical width index (lower panels) using NIH Image J 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 non-infarcted 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. [a]$P < 0.01$, 3K3A-APC alone vs. vehicle or tPA alone; [b]$P < 0.01$, tPA + 3K3A-APC vs. vehicle or tPA alone; ns, non-significant tPA alone vs. vehicle.

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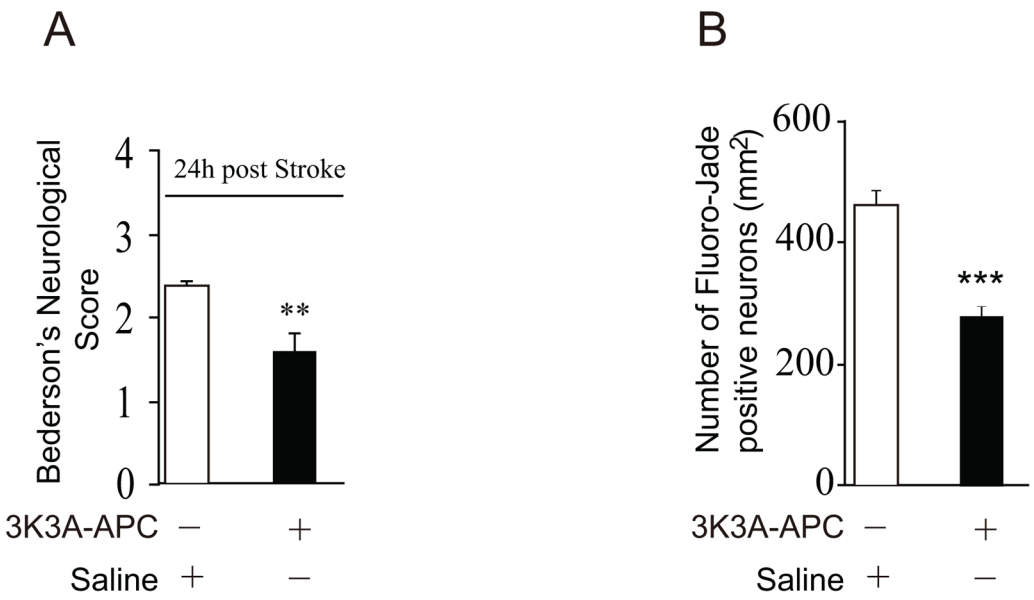

Figure 4. Effects of murine 3K3A-APC treatment alone on neurological functions and neurodegeneration post MCAo in rats

Rats administered intravenously with 3K3A-APC (0.2 mg/kg) 4 hours after MCAo were compared to saline treated animals based on Bederson's scale. **(A)** Neurological scores 24 hours post 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 post MCAo resulted in significantly reduced number of degenerated neurons in comparison to control (saline treated) animals. The degenerated neurons were counted using NIH Image J software. All values are mean ± SEM, n=8 rats per group. $^{**}P < 0.01$, 3K3A-APC vs. saline 24 hours post MCAo; $^{***}P < 0.001$, 3K3-APC vs saline.

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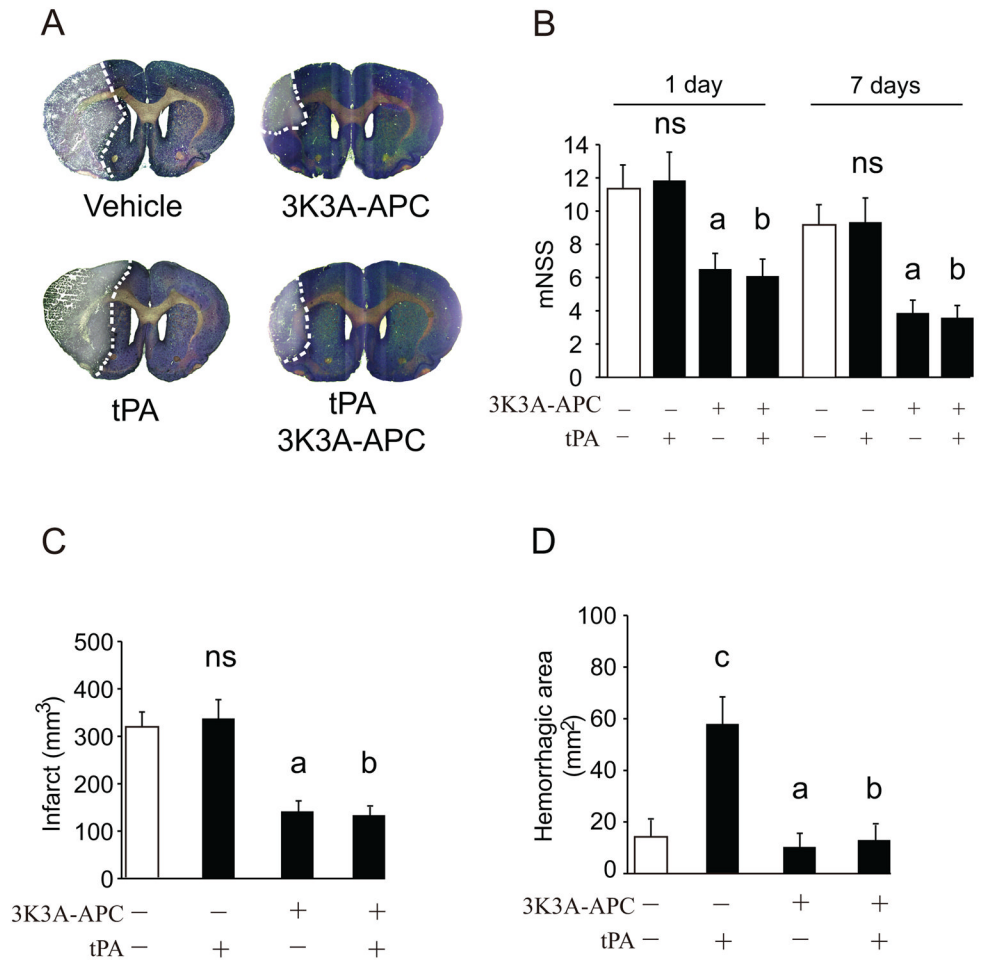

Figure 5. Effects of murine 3K3A-APC and a combined 3K3A-APC and tPA treatment on functional and neuropathological outcome and microscopic hemorrhage in young male SHR within 7 days after embolic stroke

3K3A-APC (0.2 mg/kg) and tPA (10 mg/kg) were administered intravenously 4 hours after embolic stroke. 3K3A-APC (0.2 mg/kg) was additionally injected intravenously for 3 consecutive days. (**A**) Hematoxylin and eosin staining of coronal brain sections at the level of optic chiasm of rats under different treatments was performed 7 days after stroke. (**B**) The infarct volume and (**C**) microscopic hemorrhage were determined 7 days after stroke. (**D**) Modified neurological severity score (mNSS) was determined at days 1 and 7 after stroke. All values are mean ± SD, n = 8–9 rats per group. In B–D, [a]$P < 0.01$, 3K3A-APC alone vs. vehicle or tPA alone; [b]$P < 0.01$, tPA + 3K3A-APC vs. vehicle or tPA alone; In B and D, ns, non-significant tPA alone vs. vehicle; In C, [c]$P < 0.01$, tPA alone vs. vehicle.

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Table 1

STAIR quality for 3K3-APC alone therapy and for the 3K3A-APC and tPA combination therapy using the experimental stroke scale modified by O'Collins et al.[56]

STAIR Criterion	Description	3K3A-APC	Refs	3K3A-APC/tPA	Refs
		Score		Score	
Laboratory	Focal model tested in two or more laboratories	YES	9–13	YES	12, present
Species	Focal model in two or more species	YES	9–13	YES	12, present
Health	Focal model in old or diseased animals	YES	present	YES	present
Sex	Focal model in males and females	YES	present	YES	present
Reperfusion	Tested in temporary and permanent models	YES	9–13	YES	12
Time window	Administered at least 1 hr after occlusion	YES	9–13	YES	12, present
Doses	Administered using at least 2 doses	YES	10,11	No	
Route	Using a feasible model of delivery	YES	9–13	YES	12
Endpoint	Both behavioral and histological outcomes	YES	9–13	YES	12
Long-term	Outcome measured at 4 weeks	YES	present	YES	present

STAIR criteria are not given in order of priority. Present data are used from the present study.