Published in final edited form as:

Trends Pharmacol Sci. 2012 August ; 33(8): 415–422. doi:10.1016/j.tips.2012.04.006.

The neurovascular unit and combination treatment strategies for stroke

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Abstract

Tissue plasminogen activator (tPA) administered within 4.5 hours of symptom onset restores cerebral blood flow and promotes neurological recovery of stroke patients. However, the narrow therapeutic time window and the risk of intracerebral hemorrhage after tPA treatment pose major hurdles to its clinical usage. In light of the failures of neuroprotective therapies in clinical trials, emerging concepts suggest that neuroprotection alone without restoration of tissue perfusion and vascular integrity may not be adequate for treatment of acute stroke. Here we review evidence of the use of adjuvant pharmacological agents to extend the therapeutic window for tPA via targeting the neurovascular unit and the underlying mechanisms of the combination therapy in experimental stroke.

Stroke

Stroke is a serious health problem worldwide and shows increasing prevalence as the population ages. Despite a wealth of insight into the pathogenesis of stroke, current therapies for this devastating disease are far from optimal. The only treatment approved for stroke by the USA Food and Drug Administration (FDA) is intravenous tissue plasminogen activator (tPA), which dissolves the obstructive clot to restore blood flow ¹. However, only a small percentage of stroke patients are eligible for tPA, largely due to the narrow therapeutic window ^{2–4}. In addition, thrombolysis with tPA carries a higher risk of symptomatic hemorrhagic complications, which hampers the clinical use of tPA ^{5, 6}. Thus, alternative or complementary therapeutic approaches aimed at expanding the therapeutic window for thrombolysis are of great clinical importance.

Stroke is a highly complex process which involves cerebrovasculature and parenchymal tissues through the interaction of multiple mechanisms ^{7–9}. There have been substantial advances in understanding ischemic neuronal injury. Although neuroprotection has been validated in experimental stroke, clinical trials show that many neuroprotective drugs failed to achieve desirable clinical benefit for treatment of acute stroke ^{10–14}. In addition to multifactor effects, such as clinical trial design inconsistent with preclinical studies and lack of rigorous preclinical investigation, that may have lead to failure of the clinical trials, evidence from these trials suggests that neuroprotection alone without restoration of tissue

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perfusion and vascular integrity may have compromised the therapeutic benefit of promising neuroprotective agents for treatment of acute stroke^{13–16}. The neurovascular unit is a functional and structurally interdependent multi-cellular complex, comprised of endothelial cells, the basal lamina, pericytes, astrocytes, and neurons^{17–19}. A successful therapy for acute stroke must restore the normal function of the neurovascular unit by rapidly reestablishing cerebral blood flow in the microvascular bed, preserving vascular integrity, and minimizing neuronal death. Indeed, in experimental stroke, thrombolytic agents in combination with compounds that target the neurovascular unit show synergistic effects on reduction of ischemic brain damage^{20–24}. In this review, we discuss the neurovascular protective effects of these multi-target approaches in experimental stroke and their potential as adjuvant agents in extending the therapeutic window for stroke.

Neurovascular dysfunction: a role for ischemic penumbra?

Abrupt interruption of blood supply in the brain leads to irreversible tissue damage in the ischemic core, whereas the immediate peripheral regions with less severe blood perfusion deficits contain potentially salvageable ischemic tissue - termed ischemic penumbra which gradually evolves into infarction within days in the absence of reperfusion^{25, 26}. Thus, the ischemic penumbra represents the most clinically relevant therapeutic target, and becomes the focal point of stroke research for treatment of acute stroke. It is well accepted that the evolution of ischemic brain damage is a heterogeneous and dynamic process, which is manifested by the interplay of multiple cell types through a cascade of molecular events. Although some of these events coincide spatially and temporally with the gradual recruitment of potentially salvageable tissue to the ischemic core, the others are associated with endogenous protective and repair mechanisms, and thus, generate multiple molecular penumbras of ischemic brain^{16, 27}. Emerging evidence suggests that the neurovascular unit is not only the target of these molecular events, but also actively participates in the pathogenesis of stroke. Situated at the interface between the circulating blood and brain parenchyma, endothelial cells structurally and functionally interact with basal lamina, astrocytic end-foot processes, and pericytes to form the blood-brain barrier (BBB), all of which are essential for maintaining circulatory homeostasis and neural function via facilitating neurovascular coupling, modulating vessel tone, and regulating the barrier functions of the BBB^{9, 28–31}.

Shortly after onset of focal cerebral ischemia, neurovascular dysfunction is manifested by the disruption of BBB integrity and function^{32, 33}. On the cellular level, endothelial cells rapidly convert into a pro-inflammatory/pro-thrombotic state via upregulating protease-activated receptor 1 (PAR-1) and tissue factor (TF), and matrix metalloproteinase (MMP) gene expression in the ischemic core and boundary, which facilitates inflammation and BBB disruption^{34–36}. In addition, the pericyte contraction following ischemic insult results in persistent microvascular constriction, despite successful reopening of the occluded vessel, indicating that pericytes are involved in the microvascular hemodynamic dysfunction in stroke³⁷. Furthermore, stroke induces a rapid loss of β_1 -integrin expression in the endothelial cells and astrocytes, which coincide with increased cerebrovascular permeability^{38–40}.

More recently, activation of the immune system has been implicated in neurovascular dysfunction following stroke^{41–48}. Toll-like receptors (TLRs) are the key receptors of innate immunity^{49, 50}. Following ischemic insult, the release of damage-associated molecular patterns (DAMPs) from damaged tissue, including heat shock proteins, high mobility group box 1 (HMGB1), and fibrinogen, trigger the activation of TLRs. The TLRs in turn signal through transcription factors NF- κ B and activator protein-1 (AP-1) to induce the expression of genes encoding for inflammation and apoptosis^{51, 52}(Figure 1). In the

cerebral endothelial cells, the activation of TLRs following oxidative stress causes downregulation of tight junction proteins⁵³. In addition, mice with a defective TLR4 gene have reduced MMP-9 expression and are less susceptible to ischemic brain injury, suggesting TLR4 signaling plays a crucial role for MMP-9 induction and neurovascular injury⁵⁴. Importantly, in a rat model of embolic stroke, the upregulation of interleukin-1 receptor-associated kinase-1 (IRAK-1), an important component of the TLR/NF- κ B signaling pathway, is associated with BBB disruption and increased brain hemorrhage⁵⁵. Thus, experimental evidence indicates that stroke induces a rapid BBB disruption through multiple cellular and molecular events. In parallel, the importance of neurovascular dysfunction in the pathogenesis of stroke has also been demonstrated in stroke patients. Recent studies in human brain tissues show elevated levels of MMP-9 in microvascular endothelium isolated from the ischemic area, and increased numbers of MMP-9-positive neutrophils accumulated at the perivascular areas, which co-localized with microvessel disruption and hemorrhagic transformation in the ischemic brain^{33, 56, 57}. In patients with acute middle cerebral artery (MCA) stroke, high levels of interleukin-10 (IL-10) and tumor necrosis factor- α (TNF- α), as well as low levels of IL-6 and active MMP-9 are associated with clinical-diffusion mismatch-a surrogate indication of ischemic penumbra based on the assumption that ischemic penumbra is likely present in patients with severe clinical deficits and relatively small lesion volumes measured by diffusion-weighted imaging⁵⁸. Furthermore, patients with high levels of TLRs at admission are associated with poor stroke outcome and increased inflammatory response in acute stroke⁵⁹. Thus, these data provide clinical support for the hypothesis that activation of vascular disruptive mechanisms occurs acutely at the neurovascular unit, which leads to progression of brain damage and unfavorable outcomes. Owing to the intimate relationship among cellular components, neurovascular dysfunction appears to be a potential target for the prevention of secondary damage following stroke.

Perturbation of neurovascular function following stroke limits the thrombolytic effects of tPA

Successful and timely reperfusion of ischemic brain is essential to salvage the vulnerable tissue^{1, 60}. To date, intravenous (IV) thrombolysis with tPA is the only proven intervention for acute management of stroke; tPA dissolves the fibrin contained in a clot to reestablish blood flow. However, the thrombolytic effects of tPA are far from optimal. Fewer than 40% of stroke patients establish early reperfusion after IV tPA administration^{61, 62}. In addition, the risk of symptomatic hemorrhage is high in patients receiving tPA, and hemorrhage is associated with a higher mortality and worse outcome in surviving patients^{63, 64}. Due to the heterogeneous mechanisms of stroke, the thrombolytic efficacy of tPA is largely dependent on the composition of the occlusive clots and vascular conditions. Platelet-rich clots are more resistant to thrombolysis than fibrin-rich clots^{65, 66}. In addition, stroke patients with increased plasminogen activator inhibitor-1 (PAI-1) levels - the principal inhibitor of tPA - are less susceptible to thrombolytic therapy^{67, 68}. Alternative reperfusion strategies, including combined intravenous and intra-arterial thrombolysis with tPA with or without thrombectomy, ultrasound-enhanced thrombolysis, alternative thrombolytic agents (desmoteplase and tenecteplase), and combination of thrombolytic and antithrombotic agents may enhance the efficacy and safety of thrombolytic therapy. Their possible merits over IV tPA have been thoroughly reviewed elsewhere⁶⁹, and will not be discussed further.

Thus, although proper reperfusion is essential to halt stroke progression, thrombolysis with tPA after stroke has been shown to potentiate neurovascular disruption. Indeed, in experimental stroke, acute upregulation of endogenous tPA at the astrocyte and endothelial interface interacts with low-density lipoprotein-related protein (LRP), which potentiates BBB permeability by directly altering the architecture and function of the neurovascular

unit^{70, 71}. In addition, a number of experimental and clinical studies have indicated that tPA facilitates the degradation of critical components of the BBB via the activation of MMPs^{56, 72–75}. Furthermore, tPA increases oxidative stress and inflammatory responses at the neurovascular unit, which are important mechanisms in mediating reperfusion injury and damage after stroke⁷⁶. Moreover, exogenous tPA may cross the BBB without compromising the integrity of the BBB, and then in the parenchyma, tPA may stimulate excitotoxic neuronal injury^{77–81}. Thus, as a pleiotropic protein, exogenous tPA elicits neurovascular disruption and parenchymal damage, which may constrain the beneficial effects of thrombolytic therapy in stroke. By contrast, as noted above, neurovascular dysfunction after stroke activates coagulation cascade, which facilitates the accumulation of blood elements including, platelets, fibrin, leukocytes, and erythrocytes at the site of occlusion and in downstream microvessels, and therefore, may counteract the fibrinolytic effects through releasing serpins such as PAI-1^{82, 83}. Thus, these data suggest that tPA potentiates neurovascular dysfunction, and the thrombolytic effects of tPA are hampered by the activation of anti-fibrinolytic pathway at the site of vascular dysfunction following stroke.

Neurovascular aging

Advanced aging is associated with increased morbidity and mortality in stroke patients, and represents a major risk factor for stroke^{84, 85}. It is well recognized that during aging, cerebral microvasculature undergoes profound structure and functional changes, which may contribute to increased vulnerability to ischemic insults^{86, 87}. In the normal aged brain, cerebral blood flow (CBF) reduction, and BBB leakage is a widespread phenomenon^{88–90}. In addition, tight junction proteins such as claudin 5 and occludin are disassembled^{90, 91}. Several molecular mechanisms such as oxidative stress and mitochondrial dysfunction are activated in endothelial cells, astrocytes, and microglia⁹². A recent genomic profile in the aged brain reveals that TLRs, NF- κ B, and expression of pro-inflammatory genes are upregulated, which implicates the disruption of innate immunity during aging^{93, 94}. In experimental stroke, a recent study shows that aging potentiates BBB permeability detected by quantitative MRI, which is associated with increased claudin 5 and occludin disassembly. tPA treatment results in an early BBB disruption and lesion expansion, indicating tPA exacerbates BBB disruption and tissue demise in the aged rat after stroke⁹⁰. Thus, advanced aging has a great impact on the neurovascular function. As the thrombolytic agent may render further detrimental effects on the already compromised neurovascular function, the use of the aged animal is mandatory for preclinical evaluation of potential neuroprotective agents.

Combination approaches targeting neurovascular units

Neurovascular dysfunction following stroke poses a major hurdle for thrombolytic therapy. Given the complexity of the cellular-biological mechanisms in the pathogenesis of neurovascular dysfunction following stroke, adjuvant pharmacological agents with multi-complementary mechanisms on neurovascular protection may enhance the efficacy of thrombolytic therapy without adversely compromising the integrity of the BBB. In experimental stroke, several pharmacological agents with multi-targeted neurovascular protective effects promote the efficiency and safety of thrombolysis with tPA.

Statins

Statins, also known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are a class of compounds that have pleiotropic effects well beyond their lipid lowering actions⁹⁵. In cardiovascular disease and cerebral ischemia, statins have been shown to exert potent vascular protective effects including: improvement of endothelial

dysfunction, inhibition of inflammatory and coagulation cascades, increased nitric oxide synthesis, antioxidant properties, and immunomodulatory actions^{96–98}. Given their direct mechanisms of action on neurovascular units, statins may represent the ideal adjuvant agents for adjuvant thrombolytic therapies. In a rat model of embolic stroke, combination treatment with atorvastatin and tPA extended the therapeutic window for tPA to at least 4 h after stroke onset²². The neuroprotective effects of the combination treatment are associated with the enhancement of cerebral vascular patency and integrity, whereas tPA monotherapy exacerbates cerebral vascular disruption, indicating that statins provide protection from ischemic brain damage, largely attributed to the cerebrovascular protective mechanisms^{20, 22}. Indeed, atorvastatin blocked tPA-upregulated PAR-1 and TF in cerebral endothelium, which are key modulators of thrombus formation. In addition, atorvastatin abolished tPA-induced aggravation of BBB disruption via downregulating endothelial MMP-2 and MMP-9 expression. Moreover, although tPA has been shown to potentiate NMDA receptor mediated excitotoxic neuronal death – the principal mechanisms of neuronal death in ischemic brain⁹⁹ – statins protect against NMDA-induced neuronal damage¹⁰⁰. In primary rat cortical astrocytes, simvastatin suppresses tPA-induced upregulation of MMP-9 by inhibiting the Rho kinase pathway¹⁰¹. Treatment with rosuvastatin completely abolished tPA-induced aggravation of ischemic brain damage in mice after stroke¹⁰². Thus, statins protect against the tPA-mediated neurovascular unit dysfunction, and thereby extend the therapeutic window for stroke. The efficacy of statins in lowering the risk of cerebrovascular events is now well established^{103, 104}. However, only a few clinical studies addressed the question of whether statins may improve stroke outcome following thrombolytic therapy, and the results are controversial¹⁰⁵. In an observational study of 145 patients, Alvarez-Sabin *et al.* indicated that prior statin treatment provides long-term functional benefits in stroke patients receiving intravenous tPA without increasing the incidence of hemorrhagic transformation¹⁰⁶. However, Meier *et al.* analyzed 311 patients treated with intra-arterial urokinase, in which prior statin use (55 patients) is associated with a higher frequency of hemorrhagic transformation without influencing the clinical outcome¹⁰⁷. Moreover, others have reported that statin use in stroke patients treated with tPA is not associated with favorable clinical outcome, and did not increase the risk of hemorrhagic transformation^{108, 109}. A recent retrospective study on 178 patients showed that statin treatment started within 24h after intravenous thrombolysis improves stroke outcome, whereas prophylactic statin treatment is associated with increased the risk of hemorrhagic transformation¹¹⁰. Thus, the clinical feasibility of using statins as an adjuvant agent for thrombolytic therapy requires further rigorous investigation.

Proteasome inhibitors

The ubiquitin-proteasome pathway is an important pathway for intracellular protein degradation¹¹¹. This pathway regulates central mediators of coagulation and inflammation that are fundamental mechanisms in the development of neurovascular dysfunction after stroke¹¹². Proteasome inhibitors target the ubiquitin-proteasome pathway, and they have been shown to provide significant therapeutic benefit in experimental stroke. Two different classes of proteasome inhibitor have been employed in the treatment of experimental stroke. Velcade (bortezomib, PS-341) is a dipeptidyl boronic acid, which has been clinically approved and well characterized for the treatment of patients with relapsed multiple myeloma^{113, 114}. PS-519 (MLN519) is a lactacystin derivative, which demonstrates anti-inflammatory actions in a variety of inflammatory-related pathological conditions^{115–117}. Both agents are known to effectively suppress immuno-inflammatory cascade by a mechanism involving the inhibition of NF- κ B activation in animal models of stroke. In addition, proteasome inhibitors upregulate eNOS, an important regulatory enzyme that maintains endothelium-dependent vasodilatation and also mediates antithrombotic actions^{118, 119}. Moreover, proteasome inhibitors induce antioxidative enzyme expression,

which protects neurons astrocytes, and endothelial cells from oxidative stress^{118, 120–123}. Thus, proteasome inhibitors may have multiple mechanisms of action on neurovascular units that make it attractive adjuvant agents for thrombolytic therapy. In a rat model of embolic stroke, thrombolysis with tPA at 4 h after stroke increased hemorrhage transformation without reduction of cerebral infarction, but combination treatment with PS-519 and tPA at 2, 4, or 6 h after embolization reduced cerebral infarction without increasing the incidence of hemorrhage transformation¹²⁴. Similarly, the combination treatment with bortezomib and tPA enhanced vascular patency and vascular integrity, and concomitantly extended the therapeutic window to at least 6 hours after stroke without increasing hemorrhagic transformation¹²⁵. The neurovascular protective effect of the combination of bortezomib and tPA were further demonstrated in aged rats after stroke²¹. Bortezomib neutralized tPA aggravated BBB disruption via blocking the upregulation of MMP-9. In addition, bortezomib blocked the microvascular secondary thrombus formation, and thereby amplified the thrombolytic effects of tPA (Figure 2)²¹. Collectively, these studies indicate that the proteasome inhibitor acts on multiple neurovascular protective mechanisms, which not only counteract tPA potentiated BBB disruption, but also enhanced the thrombolytic effects of tPA. As an important risk factor for stroke, advanced age is associated with lower thrombolytic efficacy and an increased risk of hemorrhagic transformation, which hamper the clinical use of thrombolytic therapy^{35, 126, 127, 128}. Thus, the beneficial effects of the combination therapy in aged rats may have important clinical implications for stroke treatment.

Minocycline

Minocycline is a broad-spectrum tetracycline antibiotic, which has been widely used for the treatment of various types of bacterial infections. In addition to its well documented anti-inflammatory, anti-apoptotic, and antioxidant properties in the experimental stroke, minocycline exerts potent vascular-protective effects via suppressing stroke induced MMP activation^{129–131}. Adjuvant administration of minocycline suppresses tPA-potentiated upregulation of plasma levels of MMP-9 and hemorrhagic complications, and thereby extends the thrombolytic time window to 6 hours after embolic stroke in rats¹³². In a transient ischemic model of stroke, treatment with minocycline attenuates tPA induced MMPs activation and basal lamina protein degradation without compromising the proteolytic activity of t-PA. In light of these promising preclinical findings, the clinical efficacy of minocycline is currently under investigation. Lampl *et al*/indicated that treatment with minocycline within 24 after stroke onset is associated with better clinical outcome¹³³. More intriguingly, a recent completed early phase, open-label trial-minocycline to improve neurologic outcome in stroke (MINOS) suggests that minocycline is safe to use alone and in combination with tPA, and can effectively lower plasma MMP-9 levels in patients with or without tPA treatment^{134, 135}. Thus, the beneficial effects of minocycline in the acute management of stroke and its desirable safety profile make it an attractive candidate for use as an adjunctive therapy to thrombolysis. However, these encouraging early findings should be confirmed by further investigations.

Free radical scavengers

The excessive generation of free radicals following stroke is a major contributor of neurovascular dysfunction following stroke^{136, 137}. In experimental stroke, free radical scavengers are proven to be effective in reducing BBB disruption as well as ischemic brain damage^{138, 139}. In addition, treatment with various free radical scavengers markedly reduced t-PA-induced hemorrhage and exerted potent neuroprotective effects^{140–142}. Thus, free radical scavengers represent potential adjuvant agents for thrombolytic therapy. So far, several free radical scavengers are under various stages of clinical testing (recently reviewed in¹⁴³). Despite the promising result from SAINT-I in which acute administration of

disodium 2,4-disulphophenyl-*N*-tert-butyl nitron (NXY-059) decreased incidence of tPA-related hemorrhagic complications, pooled data from SAINT-I and SAINT-II trials failed to show meaningful clinical benefit. Recently, a multicenter, randomized, open-labeled study reported that co-administration of edaravone with low dose tPA (0.6mg/kg) is associated with early recanalization and good clinical outcome as compared with patients treated with tPA alone, indicating that the use of free radical scavengers may potentially enhanced the thrombolytic efficacy of tPA¹⁴⁴. Clinical trials free radical scavengers are ongoing and further studies are needed to determine whether free radical scavengers will benefit stroke patients with thrombolytic therapy.

Erythropoietin (EPO)

The cytokine (EPO) is a naturally occurring hormone which was originally described as a primary regulator of erythropoiesis. In the ischemic brain, EPO has been reported to induce neurovascular protection through a broad range of cellular and biological properties. EPO treatment protects against ischemia-induced BBB disruption through down-regulating Flk-1 expression and the response to the VEGF pathway¹⁴⁵. In addition, EPO attenuates inflammatory response, and exerts antiapoptotic and anti-oxidant effect in the animal model of stroke^{146–148}. However, despite the robust beneficial effects of EPO observed in experimental stroke, the clinical translation of EPO as a neuroprotective agent failed. A double-blind, placebo-controlled, randomized German Multicenter EPO Stroke Trial¹⁴⁹ reported that EPO treatment failed to improve clinical outcomes and increased mortality rate when compared with patients treated with placebo. Subgroup analysis of patients reveal that although patients receiving EPO without tPA treatment showed a better clinical outcome, a significantly increased mortality rate was found in the tPA population, suggesting a adverse EPO and tPA interaction. The detrimental effects of EPO and tPA combination were further demonstrated in experimental stroke^{55, 150}. In mice subjected to a 90 min transient MCA occlusion, EPO monotherapy reduced brain edema, whereas combination treatment with EPO and tPA exacerbated the BBB permeability and brain edema. Although the combination treatment with EPO and tPA initiated 2h after embolic stroke reduced ischemic brain damage without compromise the BBB integrity, the delayed combination treatment at 6h carries a higher risk of intracerebral hemorrhage without reduction of ischemic brain damage. Upregulation of IRAK1 and activation of NF- κ B in cerebral endothelial cells likely contribute to exacerbate neurovascular damage after the delayed combination treatment⁵⁵. These findings are consistent with the clinical finding in which a negative influence of tPA protocol violation including treatment beyond the 3h time window (16%) may have contributed to the high mortality rate in patients treated with EPO¹⁴⁹. Taken together, these studies suggest that the clinical translation of adjuvant agents with thrombolytic therapy requires preclinical assessment for their feasibility, safety, and efficacy.

Concluding remarks

In conclusion, neurovascular dysfunction after stroke is multifaceted, which may contribute to a board pathological impairment in the ischemic brain. Here, we described the effects of multi-target neurovascular protective agents in experimental stroke and their potential to enhance the beneficial effects of thrombolytic therapy while minimize the hemorrhagic complication. Although the combination of thrombolytic therapy and pharmacological agents with pleiotropic actions on neurovascular unit appears to be a rational strategy for the treatment of stroke, successful translation of therapies into the clinical will need rigorous investigation of possible detrimental interaction.

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Figure 1. Endothelial cell dysfunction and TLR signaling

In response to ischemic insult, endothelial cells express protease-activated receptor 1 (PAR-1), tissue factor (TF), and matrix metalloproteinases (MMPs). This process facilitates the accumulation of fibrin, platelet, and neutrophil, and results in microvascular obstruction. On the abluminal front, MMPs degrade neurovascular matrix, leading to acute BBB disruption. Release of endogenous ligands from damaged cells lead to the activation of TLRs, which signal through a number of adaptors and kinases, such as MyD88, IRAK, TRAF-6. These signaling cascades lead to the production of proinflammatory cytokines through the activation of transcription factors, such as NF-κB and AP-1, which contribute to neurovascular damage.

Figure 2. Effects of combination treatment with BORTEZOMIB and tPA on thrombolysis and vascular patency

Panel A shows a schematic representation of the ICA and the MCA. A large fragment of Evans blue labeled residual embolus (red) was detected within the origin of the MCA and intracranial segment of the ICA, which blocked plasma perfusion (green) from representative saline-treated rats (B). The combination treatment with bortezomib and tPA resulted in only a small fragment of residual embolus (C, red) at the origin of the MCA where was well perfused by FITC-dextran (C, green). Panels D and E are two-dimensional projections of fibrin/fibrinogen immunoreactivity (red) and vascular plasma perfusion (green) acquired from representative rats treated with saline (D), and combination of BORTEZOMIB and tPA (E). Intravascular fibrin/fibrinogen immunoreactivity was associated with the absence of plasma perfusion in saline treated rats (Arrows in D), suggesting intravascular fibrin deposition blocks plasma perfusion. The combination treatment with bortezomib and tPA reduced number of fibrin/fibrinogen immunoreactive vessels and preserved plasma perfusion compared with saline treated rats. Bars = 400 μ m for panels B and C, and 100 μ m for panels D and E.