Combination Treatment With VELCADE and Low-Dose Tissue Plasminogen Activator Provides Potent Neuroprotection in Aged Rats After Embolic Focal Ischemia

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Background and Purpose—Treatment with a selective proteasome inhibitor, VELCADE, in combination with tissue plasminogen activator (tPA) extended the therapeutic window to 6 hours in young rats after stroke. However, stroke is a major cause of death and disability in the elderly. The present study investigated the effect of VELCADE in combination with a low-dose tPA on aged rats after embolic stroke.

Methods—Male Wistar rats at the age of 18 to 20 months were treated with VELCADE (0.2 mg/kg) alone, a low-dose tPA (5 mg/kg) alone, combination of VELCADE and tPA, or saline 2 hours after embolic middle cerebral artery occlusion. To test the contribution of endothelial nitric oxide synthase to VELCADE-mediated neuroprotection, endothelial nitric oxide synthase knockout and wild-type mice were treated with VELCADE (0.5 mg/kg) 2 hours after embolic stroke.

Results—Treatment with VELCADE significantly reduced infarct volume, whereas tPA alone did not reduce infarct volume and aggravated blood–brain barrier disruption in aged rats compared with saline-treated rats. However, the combination treatment significantly enhanced the reduction of infarct volume, which was associated with an increase in endothelial nitric oxide synthase activity compared with saline-treated rats. Additionally, the combination treatment promoted thrombolysis and did not increase the incidence of hemorrhage transformation. VELCADE significantly reduced lesion volume in wild-type mice but failed to significantly reduce lesion volume in endothelial nitric oxide synthase knockout mice.

Conclusions—Treatment with VELCADE exerts a neuroprotective effect in aged rats after stroke. The combination of VELCADE with the low-dose tPA further amplifies the neuroprotective effect. Endothelial nitric oxide synthase at least partly contributes to VELCADE-mediated neuroprotection after stroke. 

Key Words: embolic stroke • thrombolysis

Stroke is a leading cause of death and disability worldwide, primarily affecting the elderly.1 The only Food and Drug Administration-approved treatment for acute stroke is thrombolysis with tissue plasminogen activator (tPA), which restores cerebral blood flow and improves neurological outcome in patients with acute ischemic stroke.2 However, tPA treatment is of limited use in the elderly population.1 Evidence suggests that advanced age is the most important predictor of intracerebral hemorrhage in patients receiving thrombolytic therapy.1 In addition, increasing age is associated with increased in-hospital mortality in patients treated with tPA.4 Moreover, advanced age is associated with elevated prothrombotic factors and impaired fibrinolytic activity, which may hamper the efficacy of thrombolysis.5 Thus, the alteration of vascular pathology in aging individuals may provoke stroke-initiated adverse cerebral vascular events such as secondary thrombosis and blood–brain barrier (BBB) disruption, which limit the clinical use of tPA.6,7 Therefore, a complementary approach aimed at promoting cerebrovascular integrity and blocking adverse cerebral vascular events may increase the thrombolytic efficacy of tPA and reduce tPA-induced hemorrhagic transformation, and, thereby, may make thrombolytic therapy more accessible to the aged population.

The ubiquitin–proteasome pathway is the principal mechanism for the turnover of many short-lived regulatory proteins,8 and many of these proteins function as central mediators of thrombosis and BBB permeability that are fundamental mechanisms in the development of adverse vascular events after stroke. Administration of proteasome inhibitors attenuate vascular thrombogenic and inflammatory events and exert a neuroprotective effect in experimental stroke.9–11 To mimic the clinical situation, we therefore propose to examine the neuroprotective effect of

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VELCADE alone and in combination with a low-dose tPA in aged rats subjected to embolic stroke. We hypothesized that the combination of VELCADE and tPA attenuates adverse cerebral vascular thrombogenic events and BBB disruption and thereby provides more potent neuroprotection compared with individual therapy in aged rats after embolic stroke. We also tested the hypothesis that endothelial nitric oxide synthase (eNOS) contributes to the therapeutic benefits observed with the combination therapy.

Materials and Methods
All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. All outcome measurements were performed by observers blinded to the treatments.

Experimental Groups
Male Wistar rats (Charles River Laboratories France, L’Arbresle Cedex, France) at the age of 18 to 20 months were subjected to embolic middle cerebral artery (MCA) occlusion. Ischemic rats were randomly divided into the following groups: (1) VELCADE (Millennium Pharmaceuticals) alone (n=14); (2) tPA (generously provided by Genentech) alone (n=14); (3) combination of VELCADE with tPA (n=17); or (4) saline (n=18). Treatment with VELCADE at a dose of 0.2 mg/kg results in an 80% inhibition of proteasome activity, which is associated with reduction of cerebral infarction and functional deficits in young rats after stroke.9,10 Administration of tPA at a dose of 10 mg/kg improves cerebral reperfusion and reduces ischemic brain damage when administered within 2 hours in young rats after embolic stroke.11 In a pilot experiment, the aged rats that received a full-dose tPA (10 mg/kg) had an unexpectedly high early mortality rate of 67% (4 of 6), which precludes use of the full-dose tPA at a dose of 10 mg/kg.

Combination Treatment With VELCADE and tPA

Mortality
The mortality rates were 42%, 38%, 41%, and 32% for rats treated with saline, VELCADE alone, tPA alone, and a combination of VELCADE and tPA, respectively. All of the rats that died did so within 48 hours after MCA occlusion, and autopsy revealed that these animals had massive ipsilateral hemispheric swelling. No significant differences were detected among the groups. Rats that died were excluded from further evaluation.

Results

Quantitative Measurements of Residual Embolus and Cerebral Vascular Perfusion
To quantify the change of an embolus, Evans blue-labeled clot, which emits red fluorochrome, was used to induce MCA occlusion. Residual embolus at the origin of the MCA was measured 24 hours after stroke, as previously described.14

Immunohistochemistry

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To examine cerebral microvessel perfusion, fluorescein isothiocyanate (FITC) dextran (Sigma; 50 mg/rat, intravenously) was administered 24 hours after stroke. Rats were euthanized 5 minutes after FITC–dextran injection. FITC–dextran perfusion was measured as previously described.10

eNOS Activity Assay
eNOS activity was measured by monitoring the conversion of [14C] arginine to [14C] citrulline with the NOS Assay Kit (Cayman Chemical, n=3/group) according to the manufacturer’s instructions.

Statistical Analysis
Two-way analysis of variance was used to test the synergistic or additive effects of the treatment combination (see Supplemental Methods for more details). Logistic regression analysis was used to test the mortality and gross hemorrhagic rates among the groups.

Histopathologic Studies
Animals were euthanized 7 days after MCA occlusion. Infarct volume and gross hemorrhagic were measured as previously described.11

Immunohistochemistry

Immunohistochemistry was performed 1 day after stroke, as previously described.10 The following primary antibodies were used in the present study: polyclonal antithrombocyte (Inter-Cell Technologies; 1:200), polyclonal antimeylperoxidase (DAKO; 1:200), mAb antiful fibrinogen/fibrin (Accurate Chemical & Scientific; 1:1000), mAb anti-matrix metalloproteinase-9 (MMP-9; Chemicon; 1:100), and mAb antienothelial barrier (Sternberger Monoclonals; 1:1000). See Supplemental Methods (available at http://stroke.ahajournals.org) for 3-dimensional image acquisition and analysis.
Combination treatment with VELCADE and tPA significantly reduced the number of vessels with intravascular fibrin and platelet deposition and myeloperoxidase accumulation in the ipsilateral hemisphere compared with the saline treatment (Figure 3). Treatment with VELCADE alone significantly reduced the number of vessels with intravascular and platelet deposition and neutrophil accumulation but failed to reduce the fibrin immunoreactive vessels compared with the control (Figure 3). However, monotherapy with tPA had no effects on intravascular fibrin and platelet deposition as well as neutrophil accumulation compared with the saline treatment (Figure 3). These data indicate that a combination of VELCADE and tPA reduces microvascular thrombus formation.

Treatment with VELCADE alone and in combination with tPA significantly increased microvascular areas perfused with FITC–dextran in the ipsilateral hemisphere compared with the areas in rats treated with saline and tPA alone (Figure 3). However, treatment with tPA alone resulted in a trend toward decreased microvascular areas perfused with FITC–dextran compared with saline-treated rats (Figure 3). Collectively, the combination treatment significantly reduced residual emboli and downstream microvascular thrombus formation and enhanced vascular patency, indicating that the combination treatment enhances thrombolysis.

The Effect of the Combination Treatment on Cerebrovascular Integrity

The large plasma protein fibrin/fibrinogen extravasates into the parenchyma after stroke, which indicates BBB disruption.7 In the present study, treatment with tPA alone significantly increased the number of vessels with fibrin/fibrinogen extravasation compared with the control. Conversely, treatment with VELCADE alone or in combination with tPA significantly reduced the number of vessels with extravascular fibrin/fibrinogen deposition compared with the saline and tPA alone groups (Figure 4). A significant treatment interaction (subadditive effect) in reduction of fibrin extravasation was detected in rats treated with combination of VELCADE and tPA.

MMP-9 specifically degrades collagen Type IV and contributes to BBB breakdown after stroke.15 Quantitative measurements revealed that tPA alone significantly in-
Increased MMP-9 immunoreactive vessels, which was associated with a significant reduction in collagen Type IV immunoreactive area in the ipsilateral hemisphere compared with saline-treated rats (Figure 4). In contrast, combination treatment of VELCADE and tPA significantly reduced the number of MMP-9 immunoreactive vessels and preserved collagen Type IV immunoreactive area compared with the saline and tPA alone group (Figure 4).

Figure 3. Microvascular thrombosis and patency. A–B, Two-dimensional projections of fibrin/fibrinogen immunoreactivity (red) and vascular plasma perfusion (green) acquired from representative rats treated with saline (A) and a combination of VELCADE and tPA (B). Intravascular fibrin/fibrinogen immunoreactivity was associated with the absence of plasma perfusion (arrows in A–B), suggesting intravascular fibrin deposition blocks plasma perfusion. C–D, Double immunostaining of thrombocytes (green) and EBA (red) in representative rats treated with saline (C) and a combination of VELCADE and tPA (D). Quantitative data (E) show that the combination treatment significantly reduced the number of fibrin/fibrinogen immunoreactive vessels compared with the monotherapy. F–G, Treatment with VELCADE alone and in combination with tPA significantly reduced the number of vessels with platelet deposition (F) and neutrophil accumulation (G). H–K, Microvascular FITC–dextran perfusion from representative rats treated with saline (H–I) or combination of VELCADE and tPA (J–K) 1 day after stroke. L, Percentage of microvascular area perfused with FITC–dextran within the ipsilateral hemisphere. N=4/group. Bar=100 μm for A–D. Bar=1 mm for H–K. EBA indicates endothelial barrier antigen.
These data suggest that VELCADE treatment alone and in combination with tPA ameliorates BBB disruption, whereas monotherapy with tPA exacerbates BBB disruption.

Combination Treatment With VELCADE and tPA Increases eNOS Activity

To examine whether the beneficial effects of the combination treatment are associated with an increase of eNOS activity, an eNOS activity assay was performed. Combination treatment with VELCADE and tPA significantly \((P<0.05)\) increased ipsilateral eNOS activity compared with saline-treated rats (Figure 5A).

Knockout of eNOS Gene Reduces the Neuroprotective Effect of VELCADE

There were no significant differences in cerebral infarct volumes between wild-type and eNOS knockout mice after saline treatment. Treatment with VELCADE resulted in a robust reduction (57%) of lesion volume in wild-type mice. However, in eNOS knockout mice, administration of VELCADE induced a moderate, nonsignificant reduction in lesion volume compared with the control (Figure 5B).

Discussion

The present study demonstrates that administration of a clinically approved proteasome inhibitor, VELCADE, at 2

![Figure 4. VELCADE abolishes tPA-induced BBB disruption. A–B, Double immunostaining of fibrinogen/fibrin (green) and EBA (red) in representative rats treated with tPA (A) and a combination of VELCADE and tPA (B). C–F, Double immunostaining of MMP-9 (green) and collagen Type IV (red) in representative rats treated with tPA (C–D) and a combination of VELCADE and tPA (E–F). G–I, Quantitative data of extravascular fibrinogen/fibrin deposition (G), MMP-9 (H), and collagen Type IV (I) expression. N=4/group. Bar=400 \(\mu\)m. EBA indicates endothelial barrier antigen.](#)

![Figure 5. A, Effects of VELCADE and tPA treatment on brain eNOS activity assayed 24 hours after MCA occlusion (n=3/group). B, Effect of VELCADE treatment on infarct volume in eNOS knockout and wild-type mice assessed 7 days after MCA occlusion (n=6/group). EBA indicates endothelial barrier antigen.](#)
hours significantly reduced infarct volume in aged rats after embolic stroke. A combination of VELCADE and tPA neutralizes tPA aggravated BBB disruption and amplifies thrombolysis, which concomitantly resulted in a further reduction of lesion volume as compared with the monotherapy. The combination treatment significantly increased eNOS activity in aged rats after stroke, whereas the neuroprotective effect of VELCADE was attenuated in eNOS knockout mice, indicating that eNOS, at least partly, contributes to VELCADE-mediated neuroprotection in aged rats after embolic stroke.

In the present study, we found that the aged rats in the control group had a mortality rate (42%), which is substantially higher than the mortality rate (less than 10%) in young rats. All the aged animals that died within 48 hours had massive ipsilateral hemispheric swelling, indicating severe cerebral edema and brain damage. These findings are consistent with published studies on aged ischemic rats from others.

Thrombolysis with tPA at a dose of 10 mg/kg improves cerebral perfusion and reduces ischemic brain damage when administered within 2 hours after stroke onset in young rats. In the present study, administration of tPA at half of the commonly used dose failed to significantly reduce residual clots nor restore cerebral plasma perfusion in aged rats 2 hours after stroke onset. Given the high plasma levels of plasminogen activator inhibitor-1 as well as age-associated elevation of prothrombotic and antifibrinolytic factors, we do not anticipate a low-dose tPA will achieve successful thrombolysis. However, even at the subtherapeutic dose, tPA increased the microvascular MMP-9 level, which was associated with reduction of vascular collagen IV and fibrin extravasation, indicating tPA exacerbates BBB disruption in aged rats after stroke. Elevation of MMP-9 is associated with BBB damage and hemorrhagic transformation after stroke. Our results are in agreement with clinical findings that age is a significant risk factor for cerebral hemorrhage after thrombolysis in patients with stroke. Treatment with a proteasome inhibitor, MG132, significantly reduced endothelial cell MMP-9 secretion under conditions relevant to brain ischemia. Our previous studies indicated VELCADE blocked tPA-induced upregulation of MMP-9 in young rats after stroke. In the present study, administration of VELCADE completely neutralized tPA-induced upregulation of MMP-9, synergistically reduced fibrin extravasation, and did not increase the incidence of hemorrhagic transformation. Thus, our data suggest that VELCADE ameliorates tPA-exacerbated BBB disruption by suppressing MMP-9.

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Stroke elicits vascular dysfunction, which triggers a cascade of secondary thrombogenic events and thereby exacerbates vascular perfusion deficits and BBB disruption. Our previous studies indicated that VELCADE treatment targets vascular prothrombotic events and thereby enhances the thrombolytic window of tPA to 6 hours in young rats after stroke onset. However, the normal aging process is associated with the increase of clotting factors, which along with an age-associated angiopathy, shift the hemostatic balance toward a higher thrombotic tendency and delayed fibrinolysis. Therefore, the thrombolytic efficacy of VELCADE and tPA in the treatment of stroke may decline with aging. In the present study, the combination treatment significantly reduced the residual clot and downstream microvascular thrombosis, which is associated with increased downstream microvascular patency. Collectively, our data indicate that VELCADE amplifies the thrombolytic effects of tPA and neutralizes tPA aggravated BBB disruption, and the combination treatment provides synergistic neuroprotective actions in aged rats after stroke. Our findings are supported by observations from other groups in the aged ischemic rat that early disruptions of the BBB contribute to exacerbation of ischemic damage and indicate that the early recanalization after the combination treatment of VELCADE and tPA reduces disruption of the BBB, which contributes to reduction of ischemic damage.

Upregulation of eNOS reduces cerebral vascular thrombogenic events and increases cerebral blood flow after stroke. In addition, eNOS gene transfer inhibits MMP synthesis and secretion, which suggests that eNOS may prevent MMP-9-mediated BBB disruption. In the endothelial cell, proteasome inhibition with MG132, lactacystin, MG262, and epoxomycin enhances eNOS expression and activity and improves endothelial function. Consistently, our published data and the present study indicate that VELCADE activates eNOS in young and aged rats, whereas knockout of eNOS substantially attenuates the VELCADE neuroprotective effect in young mice. These data suggest that eNOS activated by VELCADE acts upstream to ameliorate ischemia and tPA-induced dysfunction of cerebral endothelial cells, which leads to the neuroprotective effect.

In conclusion, our data suggest that treatment with VELCADE at 2 hours exerts a neuroprotective effect in aged rats after embolic stroke. The combination of VELCADE with the low-dose tPA markedly improved the neuroprotective effect, which represents a promising approach for the treatment of stroke. Upregulation of eNOS may underlie the beneficial effects of a combination of VELCADE and tPA in the treatment of embolic stroke.

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Disclosures
None.

References


Supplemental Methods
Three-Dimensional Image Acquisition and Analysis
To identify fibrin deposition, single immunofluorescence labeling was performed on FITC–dextran-perfused vibratome coronal sections. Briefly, vibratome sections were incubated with a goat antifibrinogen/fibrin antibody (Accurate Chemical & Scientific; 1:1000) for 3 days at 4°C and were then incubated with the secondary antibody conjugated to Cy5 (Vector Laboratories). For image acquisition, 20 optical sections (842×842 μm² format in the x–y direction) along the z-axis with a 1-μm step size were acquired for each of 8 fields of view from the ischemic area using a 2-photon laser-scanning microscope (Zeiss-Axioskop-2) under a 40× objective. For quantification, numbers of vessels with intravascular fibrinogen/fibrin immunoreactivity and FITC–dextran perfusion deficits were counted on 2-dimensional projections of 3-dimensional image stacks. Data are presented as the density of immunoreactive vessels relative to the scan area (mm²).

Statistical Analysis
Two-way analysis of variance was used to test the synergistic or additive effects of the treatment combination (see Supplemental Methods for more details). The analysis began testing for tPA and VELCADE interaction followed by groupwise comparison if the interaction or main effect or tPA or VELCADE was detected at the 0.05 level. If a synergistic effect (a significant interaction) was detected at 0.05, the quantitative synergy \(((\mu_{11}−\mu_{00})−[(\mu_{10}−\mu_{00})+(\mu_{01}−\mu_{00})])\) would be estimated with a subadditive effect if \(((\mu_{11}−\mu_{00})−[(\mu_{10}−\mu_{00})+(\mu_{01}−\mu_{00})]<0\) or superadditive effects if otherwise. A subadditive or superadditive effect indicates that the effect of the combination treatment is superior to that of the combined effects of each treatment alone. Logistic regression analysis was used to test the mortality and gross hemorrhagic rates among the groups.
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