Minocycline and Tissue-Type Plasminogen Activator for Stroke
Assessment of Interaction Potential

Livia S. Machado, PhD; Irina Y. Sazonova, PhD; Anna Kozak, MS; Daniel C. Wiley, BS; Azza B. El-Remessy, PhD, RPh; Adviey Ergul, MD, PhD; David C. Hess, MD; Jennifer L. Waller, PhD; Susan C. Fagan, PharmD

Background and Purpose—New treatment strategies for acute ischemic stroke must be evaluated in the context of effective reperfusion. Minocycline is a neuroprotective agent that inhibits proteolytic enzymes and therefore could potentially both inactivate the clot lysis effect and decrease the damaging effects of tissue-type plasminogen activator (t-PA). This study aimed to determine the effect of minocycline on t-PA clot lysis and t-PA–induced hemorrhage formation after ischemia.

Methods—Fibrinolytic and amidolytic activities of t-PA were investigated in vitro over a range of clinically relevant minocycline concentrations. A suture occlusion model of 3-hour temporary cerebral ischemia in rats treated with t-PA and 2 different minocycline regimens was used. Blood–brain barrier basal lamina components, matrix metalloproteinases (MMPs), hemorrhage formation, infarct size, edema, and behavior outcome were assessed.

Results—Minocycline did not affect t-PA fibrinolysis. However, minocycline treatment at 3 mg/kg IV decreased total protein expression of both MMP-2 (P = 0.0034) and MMP-9 (P = 0.001 for 92 kDa and P = 0.0084 for 87 kDa). It also decreased the incidence of hemorrhage (P = 0.019), improved neurologic outcome (P = 0.0001 for Bederson score and P = 0.0391 for paw grasp test), and appeared to decrease mortality. MMP inhibition was associated with decreased degradation in collagen IV and laminin-α1 (P = 0.0001).

Conclusions—Combination treatment with minocycline is beneficial in t-PA–treated animals and does not compromise clot lysis. These results also suggest that neurovascular protection by minocycline after stroke may involve direct protection of the blood–brain barrier during thrombolysis with t-PA. (Stroke. 2009;40:3028-3033.)

Key Words: cerebral ischemia ■ minocycline ■ t-PA ■ matrix metalloproteinases ■ vascular protection ■ hemorrhagic transformation

The scientific community and regulatory bodies demand that all new acute treatments for ischemic stroke be evaluated for their potential to interact with the only approved therapy, tissue-type plasminogen activator (t-PA). Intravenous treatment with t-PA within 3 hours of stroke onset has been shown to be beneficial in achieving better outcomes,1 and recent information suggests that carefully selected patients may benefit when treated even up to 4.5 hours after the onset of symptoms.2 However, there is still a great need to develop treatments that complement and enhance the safety and efficacy of t-PA.

Recent data have shown that t-PA has deleterious effects that are independent of its fibrinolytic activity and that t-PA leads to increased chances of hemorrhagic transformation by amplifying the matrix metalloproteinase (MMP) cascade triggered by ischemic damage in the brain.3 MMP-2 (72 kDa) and -9 (92 kDa) have been shown to be elevated early after experimental stroke in rats,4,5 and the formation of edema and hemorrhagic transformation associated with thrombolysis with t-PA have been linked to MMPs.6,7 MMP inhibition might therefore represent a target for decreasing the risks of thrombolysis.10

Minocycline treatment has been shown to decrease the disruption and leakage of the blood–brain barrier11 and attenuate the enzymatic activity of the proteolytic enzyme MMP-9 after stimulation with vascular endothelial growth factor.12 Furthermore, minocycline has been shown to decrease the microvascular permeability associated with...
MMP-2 and MMP-9 activity.\textsuperscript{13} Our previous studies showed that postinjury minocycline treatment decreased the activation of MMPs after temporary cerebral ischemia.\textsuperscript{14}

Although minocycline is a promising neuroprotective strategy, a thorough investigation of its interaction with t-PA after stroke has not been pursued. Minocycline could reduce t-PA’s fibrinolytic activity through its enzyme-inhibitory effect. However, it could also prevent reperfusion-induced MMP activation and vascular damage. This study aimed to determine whether the interaction of minocycline with t-PA was significant in vitro and in an experimental model of stroke.

**Materials and Methods**

**t-PA In Vitro Clot Lysis and Amidolytic Activity**

T-PA activity was measured by in vitro fibrinolytic and amidolytic assays. For the fibrinolytic assay, whole blood was obtained from 4 healthy individuals. This study was approved by the human assurance committee of the Medical College of Georgia. Whole blood (50 µL) from healthy individuals was mixed with trace amounts of \(^{125}\)I-labeled human fibrinogen (100,000 counts per minute) and clotted. The formed clots were washed, suspended in plasma (1 mL), and clotted in a water bath at 37°C. A wide range of clinically relevant concentrations of minocycline (0 to 30 µg/mL) was added to the supernatant, and clot lysis was immediately initiated by 2 nmol/L t-PA (Activase, Genentech). The degree of fibrinolysis was measured at various times of incubation by counting the percent soluble \(^{125}\)I-fibrin degradation products.\textsuperscript{15,16} The kinetic parameters of amidolyis were measured in the presence or absence of minocycline with H-\(\text{D}-\text{Ile}-\text{D}-\text{Val}-\text{Lys}-\text{Arg}-\text{NH}_2\) (Griffonia simplicifolia) dihydrochloride (S-2288, Chromogenic) as the substrate. T-PA (100 nmol/L) was added to the microtiter plate containing the assay buffer (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl; pH 8.4), minocycline (30 µg/mL), and S-2288 (150 to 1500 µmol/L) at 37°C. The generation of amidolytic activity was measured at 405 nm for 7 minutes in a microplate reader (Synergy HT, Bio-Tech). The data were plotted as velocity of \(p\)-nitroanilide release over substrate concentration and analyzed by hyperbolic curve fitting with GraphPad Prism software.\textsuperscript{17}

**Animal Procedures and Experimental Stroke**

The institutional animal care and use committee of the Veterans Affairs Medical Center approved our study protocol. Male Wistar rats weighing 270 to 300 g were purchased from Charles River Laboratories (Wilmington, Mass.). Cerebral ischemia was induced by intraluminal suture occlusion of the middle cerebral artery for 3 hours.\textsuperscript{14} Immediately before removal of the suture, the jugular vein was exposed and the tip of a rat jugular vein catheter (Braintree Scientific R-JVC) was inserted into the lumen. After the suture was removed, the jugular catheter was connected to polyethylene-50 tubing and flushed with 1 mL of heparinized saline, followed by a bolus injection containing 10% of the t-PA dose, and a 20-minute infusion was begun (Harvard Apparatus Infusion Pump). Animals were assigned to 1 of 4 groups: control (saline treatment only); t-PA alone (10 mg/kg); t-PA plus minocycline (Sigma Aldrich Co; 3 mg/kg IV, jugular vein); and t-PA plus minocycline given intraperitoneally. Minocycline 45 mg/kg IP was injected 5 minutes after the onset of reperfusion, and both IV and IP groups received an intraperitoneal dose 12 hours later. Sacrifice with either saline perfusion (infarct size and hemoglobin) or flash-freezing (molecular markers) of brain tissue occurred 24 hours after stroke.

**Gelatin Zymography and MMP Immunoblotting**

After extraction, the brain was washed with ice-cold phosphate-buffered saline and placed in a coronal matrix to be sliced and homogenized, as previously described by Heo and collaborators\textsuperscript{8} and Machado et al.\textsuperscript{14} Gelatin zymography and MMP-2 and -9 immunoblotting were performed as reported in our previous study.\textsuperscript{14} The bands were quantified with the use of GelPro image analysis software.

**Collagen Type IV and Laminin-\(\alpha_1\) Slot-Blot Analysis**

The basal lamina components, laminin-\(\alpha_1\) and collagen type IV, were used to determine the health of the blood–brain barrier in the experimental animals. Both collagen IV and laminin proteins were studied with slot blotting and semiquantification by densitometric analysis. The same brain tissue homogenate used for MMP analysis was used. Nitrocellulose membranes were used. Samples were loaded into the Bio-Dot apparatus (Bio-Rad Laboratories) and a slow vacuum was applied. Collagen type IV antibody (rabbit polyclonal anti-collagen IV, Santa Cruz Biotechnology) and laminin-\(\alpha_1\) (goat polyclonal anti-laminin-\(\alpha_1\), Santa Cruz Biotechnology) were used. The secondary antibodies, anti-rabbit IgG and anti-goat IgG horseradish peroxidase–conjugated antibodies, were used, respectively. The membranes were developed and exposed to autoradiography films (Hyblot CL, Denville Scientific Inc). Semiquantification of the bands was performed with the use of GelPro image analysis software.

**Infarct Size and Edema Determination**

The infarct volumes were measured on 2,3,5-triphenyltetrazolium chloride–stained brain slices, as previously described.\textsuperscript{18} The image of the slices was captured and analyzed with Zeiss KS300 software. Total infarct volume was reported as percent volume of the total ischemic hemisphere. Edema was quantified by the percent difference in volume between the stroke and contralateral hemispheres.

**Hemorrhage Formation and ELISA for Hemoglobin**

The presence of visible hematoma or hemorrhagic transformation was recorded, and quantification of hemorrhage was done by assessing the hemoglobin content in the tissue after complete perfusion of the brain. This was accomplished by an ELISA method for hemoglobin, as has been previously reported.\textsuperscript{19}

**Neurologic Examination**

All animals were examined for motor function immediately before reperfusion and before sacrifice. The Bederson method\textsuperscript{20} and paw grasp test were used. The paw grasp test uses a scale of 0 to 3 and determines the use and grasping strength of the ipsilateral forelimb. The occurrence of death or near-death (unable to perform tests) was also recorded.

**Data Analysis**

To examine differences in various outcome measures between treatment groups, \(\chi^2\) tests (when the variable was categorical) and 1-way ANOVA (when the variable was continuous) were performed. Because not all post hoc pairwise comparisons were warranted between treatment groups, a Bonferroni adjustment to the overall \(\alpha\) level for the number of post hoc comparisons was used. All statistical analyses were performed with SAS 9.1.3, and overall statistical significance was assessed at an \(\alpha\) level of 0.05.
Results

**t-PA Activity**

Kinetic studies were performed to determine whether minocycline might directly affect t-PA proteolytic activity. The kinetic parameters of cleavage of the tripeptide substrate S-2288 at pH 7.4 and 37°C by t-PA are shown in Table 1. Minocycline (30 μg/mL) did not change either the apparent Michaelis-Menten constant (K_m) or the catalytic constant (K_cat). Thus, minocycline does not affect the amidolytic efficiency of t-PA. To test the fibrinolytic activity of t-PA, an in vitro clot lysis assay was used. In the presence of different minocycline concentrations (1 to 30 μg/mL), the rate of clot lysis by t-PA (2 nmol/L) remained at 17%, 38%, and 55% after 35, 75, and 120 minutes, respectively, regardless of minocycline concentration (Figure 1). At no concentrations tested did minocycline decrease fibrinolysis by t-PA.

**MMP-2 and MMP-9**

Only 2 bands were detected by gelatin zymography: 85 kDa and 67 kDa, corresponding to active MMP-9 and active MMP-2, respectively. t-PA treatment during reperfusion increased the activity of both MMP-2 and MMP-9 in the brain. The minocycline-treated animals had decreased t-PA–induced exacerbation of MMP activity compared with untreated animals, but these differences were not significant (Figure 2A). Intravenous minocycline treatment, however, significantly impacted the protein content of both MMP-2 (detected at 72 kDa: F=3.88, P=0.0185) and MMP-9 (92 kDa: F=4.75, P=0.008; and 87 kDa: F=4.03, P=0.0160) bands (Figure 2B). Intravenous minocycline significantly decreased all of the bands detected compared with t-PA (P=0.0034 for MMP-2, P=0.0084 for 92-kDa MMP-9, and P=0.0001 for 92-kDa MMP-9) and 87-kDa MMP-9 compared with control (P=0.004).

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Figure 1. Impact of minocycline on fibrinolytic activity of t-PA. Lysis of blood clots was initiated by 2 nmol/L t-PA at 37°C. The amount of fibrinolysis was determined by measuring the release of soluble 125I-fibrin degradation products at various time intervals (35, 75, and 120 minutes). The fibrinolytic activity of t-PA was not affected by any concentration of minocycline tested (1–30 μg/mL). Means±SEM (n=4) are shown.

Figure 2A. Brain MMP-2 and MMP-9 activities after stroke, as measured by gelatin zymography. MMP-2 and MMP-9 were elevated by t-PA compared with ischemic saline controls. Minocycline (Mino) decreased MMP-9 activity to below control levels (*P=NS). B, Brain MMP-2 (72 kDa) and MMP-9 (87 and 92 kDa) protein contents 24 hours after stroke, as determined by immunoblotting. The intravenous minocycline group had significantly lower MMP-2, 92-kDa MMP-9, and 87-kDa MMP-9 protein contents compared with t-PA-treated animals (*P=0.0034, *P=0.001, and *P=0.0084, respectively). Vertical bars indicate SEM.
Minocycline Plus t-PA and Vascular Outcome

We measured vascular integrity according to 4 different parameters: (1) incidence of visible hemorrhagic transformation, (2) content of hemoglobin in the brain parenchyma after complete perfusion, (3) formation of brain edema, and (4) basal lamina protein degradation. Although the content of tissue hemoglobin in t-PA–treated animals was only slightly larger than that in untreated animals (not shown), the occurrence of bleeding observed macroscopically in these animal brains was 2-fold higher than in control and intravenous minocycline–treated animals \((P = 0.0190)\) (Figure 3A). Inspection of animals that died prematurely also revealed that t-PA–treated animals had developed large brain hematomas, as illustrated in Figure 3B. Although not significant, edema was slightly elevated in the t-PA group and was decreased in combination with intravenous minocycline (Figure 3C). Ischemic brains had significantly reduced collagen type IV compared with sham brains \((F = 5.96, P = 0.0011)\). t-PA treatment further reduced the amount of collagen, and combination treatment with 3 mg/kg IV minocycline preserved this protein to above that of ischemic control animals (Figure 4A; \(P = \text{NS}\)). Likewise, intravenous minocycline robustly prevented laminin-\(\alpha1\) degradation in the brain \((F = 11.28, P < 0.0001)\) compared with both stroke groups (Figure 4B).

Infarct Size and Neurologic Evaluation

t-PA increased infarct volume \((F = 4.99, P = 0.013)\) when compared with ischemic control animals (infused with saline only; \(P = 0.0035)\). Intravenous minocycline–treated animals had decreased infarct size compared with t-PA–only animals, but it remained above control levels and was not statistically significant (Figure 5A). t-PA increased mortality, and this was ameliorated by minocycline (Table 2). Intravenous minocycline acutely resulted in improvement of the Bederson score \((F = 11.16, P < 0.0001)\) and paw grasp \((F = 3.31, P = 0.0391)\) compared with t-PA alone \((P < 0.0001)\), controls \((P < 0.01)\), and the intraperitoneal minocycline group \((P < 0.01)\) (Figure 6A and 6B). Although intraperitoneal treatment with minocycline seemed to decrease the impairment, the
effect was not statistically significant, suggesting that early drug delivery is critical to achieve optimal improved functional outcome.

Discussion

This study demonstrated that the MMP-inhibiting effect of minocycline did not impair the ability of t-PA to cleave plasminogen and exert its fibrinolytic effect. This was validated in vivo by Murata and collaborators. In their study, minocycline treatment did not have a significant effect on cerebral perfusion restored by t-PA after embolic stroke in rats. However, the clot lysis effect of the treatment interaction was not studied directly.

We demonstrated in this study that postreperfusion treatment with both low-dose intravenous minocycline and high-dose intraperitoneal minocycline inhibited the MMPs that are upregulated by treatment with t-PA. These 2 drug-dosing regimens were based on previous studies that demonstrated the therapeutic efficacy of minocycline in experimental stroke. Intraperitoneal administration has been shown to achieve a delayed but constant plasma concentration. However, because systemic delivery of minocycline is delayed, we also tested whether acute delivery of minocycline (by intravenous administration) would achieve improved or comparable results. Both treatments appeared to decrease the t-PA–induced increase in MMP-9, but only the intravenously treated group achieved a significant reduction in protein expression. These findings support our previous results demonstrating that ischemia-induced MMP-2 and -9 are sensitive to minocycline inhibition and that earlier delivery of minocycline may achieve better MMP inhibition.

The major end point in our study was the formation of hemorrhage in the brain. Image analysis of macroscopic hemorrhages showed a 2-fold increase in t-PA–treated animals when compared with untreated animals. Treatment with intravenous minocycline proved beneficial. A lack of significant reduction in edema may have been due to the insensitivity of the method used. Despite this, the increase in mortality among t-PA–treated animals appeared to be ameliorated with minocycline.

Our results also showed that minocycline decreased infarct size, even after corrected for edema, and improved overall outcome as shown by the behavior tests. It could be argued that the decreased mortality and improved behavior outcome in the combination therapy group derived from minocycline’s ability to decrease lesion volume rather than its direct protection of the vasculature. However, because hemorrhage formation has been shown to be directly related to increased mortality and because minocycline prevented basal lamina degradation, it is likely that MMP inhibition and vascular protection by minocycline contributed at least par-

Table 2. Mortality Rate

<table>
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<th>Group</th>
<th>Stroke Saline</th>
<th>t-PA</th>
<th>t-PA+Mino</th>
<th>t-PA+Mino</th>
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<td>n=11</td>
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<td>Dead</td>
<td>2 (8%)</td>
<td>7 (25%)</td>
<td>2 (18%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>Mortality rate</td>
<td>3 times higher</td>
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Mortality was 3 times higher in t-PA–treated animals compared with both stroke saline control animals and early-delivery minocycline (Mino)-treated (3 mg/kg IV) animals, although it was not statistically significant. When minocycline was delivered late (through intraperitoneal injection), mortality was similar to that of the t-PA–treated group.

Figure 5. A, Infarct size after correction for edema. t-PA significantly increased infarct size (P=0.0035). Adjuvant treatment with 3 mg/kg IV minocycline (Mino) decreased infarct size. Vertical bars indicate SEM. B, Photographs of representative brain sections of each of the treatment groups.

Figure 6. Effect of treatment on behavioral outcome. A, Improvement in the Bederson scale of animals treated with intravenous minocycline (Mino) compared with t-PA–only–treated animals (P=0.0001). B, The t-PA+intravenous minocycline treatment group performed better in the paw grasp task (P=0.0391). Vertical bars in all graphs represent SEM.
tially to the decreased mortality and overall protection after acute ischemic stroke.

Minocycline seems a logical addition to reperfusion therapy with t-PA in acute ischemic stroke. Although known as an inhibitor of proteases, minocycline in a wide range of clinically relevant concentrations does not negatively impact the ability of t-PA to lyse clots in vitro. In addition, minocycline’s pleiotropic effects in the brain include structural protection of the vasculature to prevent leakiness and hemorrhagic transformation. Because t-PA has the potential to exert multiple negative actions in the brain vasculature—either directly, or indirectly via MMPs or fibrin degradation products25—combination therapy is likely to at least be additive in benefit.

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Disclosures
Tissue plasminogen activator was a gift to the laboratory of Dr. Fagan.

References
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