Effect of a New Inhibitor of the Synthesis of 20-HETE on Cerebral Ischemia Reperfusion Injury

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Background and Purpose—Arachidonic acid that is released following cerebral ischemia can be metabolized to 20-hydroxyeicosatetraenoic acid (20-HETE). 20-HETE is a potent vasoconstrctor that may contribute to ischemic injury. This study examined the effects of blockading the synthesis of 20-HETE with TS-011 on infarct size after transient occlusion of the middle cerebral artery (MCAO) of rats and after thromboembolic stroke in monkeys.

Methods—Rats were treated with TS-011 or vehicle at various times after MCAO. Infarct size was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining and plasma levels of 20-HETE were determined by liquid chromatography mass spectrometry (LC/MS). The effect of TS-011 on infarct size was also studied in monkeys after introduction of a clot into the internal carotid artery.

Results—Plasma levels of 20-HETE increased after MCAO in rats. TS-011 (0.01 to 1.0 mg/kg per hour) reduced infarct volume by 40%. Chronic administration of TS-011 for 7 days reduced neurological deficits after MCAO in rats. TS-011 given in combination with tissue plasminogen activator also improved neurological outcome in the stroke model in monkeys.

Conclusion—These results suggest that blockade of the formation of 20-HETE with TS-011 may be useful for the treatment of ischemic stroke. (Stroke. 2006;37:1307-1313.)

Key Words: 20-HETE ☐ brain injury ☐ cytochrome P450 ☐ ischemic stroke

Each year 750,000 strokes occur in the US. A large number of the survivors experience permanent neurological damage that impacts the quality of life. The annual cost for the treatment of stroke victims is over 50 billion dollars per year. Clearly, there is a need for better therapies for ischemic stroke that minimize neurological damage. The most common model of ischemic stroke involves transient occlusion of the middle cerebral artery (MCA) in rodents. Cerebral blood flow (CBF) falls by >80% in the ischemic core and reduced to a lesser extent in the surrounding cortical tissue termed the penumbra. Irreversible neuronal damage can be minimized if a critical level of perfusion is restored within 2 hours after the onset of the stroke.1 Indeed, early intervention with the thrombolytic agent tissue plasminogen activator (t-PA) to restore CBF, improves clinical outcomes after ischemic stroke.2

Recent studies have indicated that arachidonic acid (AA) is released in cerebrospinal fluid (CSF) after cerebral ischemia.3 AA can be metabolized by cytochrome P450 (CYP) enzymes in cerebral arteries to the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE).4 20-HETE plays an important role in the regulation of cerebral vascular tone by regulating the open state probability of K⁺ channels.5 Blockade of the formation of 20-HETE attenuates myogenic tone, autoregulation of CBF and cerebral vascular responses to both vasodilators6,7 and vasoconstrictors.8,9 There is also evidence that 20-HETE plays an important role in the development of cerebral vasospasm after subarachnoid hemorrhage (SAH).9 In this regard, the levels of 20-HETE increase in CSF after SAH,10,11 and blockade of the synthesis or vasoconstrictor actions of 20-HETE prevents the fall in CBF after SAH in rats.12 and reverses delayed vasospasm in rat and dog models.13,14 Thus, it is possible that increased production of 20-HETE after ischemic stroke may contribute to brain injury by opposing the recruitment of collateral flow to the ischemic penumbra. On the other hand, 20-HETE also inhibits Na⁺, K⁺-ATPase activity, and activates a number of the intracellular signaling pathways.4 These actions may contribute to death of neurons after ischemic stroke by promoting cell swelling, rupture of intracellular organelles, release of proteolytic enzymes and the loss of mitochondrial integrity and apoptosis. Thus, the present study examined the effects of a new selective inhibitor of the synthesis of 20-HETE, TS-011 (N-(3-...
Chloro-4-morphlin-4yl)phenyl-N’-hydroxyimido formamide)\(^{15}\) on infarct volume and the development of neurological deficits after transient occlusion of the MCA in rats and a thromboembolic model of stroke in monkeys.

**Materials and Methods**

All procedures involving animals were reviewed by the Animal Care Committee of Taisho Pharmaceutical Co, Ltd, and were performed according to the guidelines published by the National Institutes of Health and the Japanese Experimental Animal Research Association.

**MCA Occlusion Model in Rats**

Studies were performed on 323 male Wistar rats (9 weeks old: Japan SLC Inc, Shizuoka) weighing 190 to 220 g. Anesthesia was induced with 3% halothane and maintained using 1% halothane during the experiment. Rectal temperature was maintained at 37°C. A PE-50 catheter was implanted in the femoral artery and vein for measurement of mean arterial pressure and infusion of drugs. Blood samples were collected before and 2 hours after MCA occlusion (MCAO) for measurement of plasma glucose level, pH, pCO\(_2\) and pO\(_2\).

Transient occlusion of the MCA (t-MCAO) was induced by intraluminal filament method as previously described.\(^{16}\) An 18-mm-long piece of 4-0 nylon suture coated with silicon was introduced into the right internal carotid artery to occlude the origin of the right MCA. Anesthesia was withdrawn and successful occlusion judged by the appearance of hemiparesis. One hour after occlusion of the MCA, the rats were reanesthetized and the suture withdrawn to allow reperfusion.

**Assessment of Neurological Deficits in t-MCAO Rats**

Neurological deficits were assessed 1, 3, 5 and 7 days after t-MCAO. Sensory motor function was tested as previously described.\(^{17,18}\) Each rat was scored for the postural reflex, visual tracking, and tactile and proprioreceptive responses. A normal response was given a score of 0 and a rat with a severe deficit received a score of 2. A composite score for the 8 tests was calculated for each animal. General motor and vestibular function was tested using a rota-rod and a beam-walking test. The rota-rod test was performed at 10 rpm by recording the time the animal remained on the rod. The time required to cross a beam 1000-mm-long, 15-mm-wide and 300-mm high with a reward box at one end was also recorded. All of the neurological tests were done 24 hours after administration of last dose of TS-011 to ensure complete washout of the drug because the half-life of TS-011 in rats is <30 minutes.\(^{16}\)

**Measurement of Infarct Volume in t-MCAO Rats**

The rats were anesthetized with diethylether and decapitated. The brains were removed and cut into seven 2-mm-thick coronal sections using Rodent Brain Matrix slicer (RBM-4000C; ASI Instruments). The sections were immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 minutes and subsequently fixed in a 10% buffered formalin solution. Infarct areas were measured using NIH-Image analysis software (Version 1.62) and total infarct volumes were calculated as the sum of the product of the infarct areas times the thickness of the sections.

**Figure 1.** A, Dose-response curve for the effect of TS-011 on infarct volume in rat t-MCAO model of ischemic stroke. TS-011 (0.001 to 1.0 mg/kg per hour) or vehicle was infused for 1 hour immediately after the initiation of reperfusion. B, Therapeutic time window for the effect of TS-011 on infarct volume. TS-011 (0.1 mg/kg per hour) or vehicle was infused for 1 hour beginning 1, 2 or 4 hours after initiation of MCAO. *P<0.05 versus the vehicle-treated group. The numbers in parentheses indicate the number of animals studied per group.
Measurement of Plasma 20-HETE Contents in t-MCAO Rats

Plasma concentrations of 20-HETE were determined using liquid chromatography mass spectrometry (LC/MS/MS). Samples (200 μL) were spiked with 2 ng of d8-20-HETE followed by solid phase extraction with a Bond Elute C18 cartridge. Mass spectrometric analysis was performed on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) using negative electrospray ionization-MS/MS. Ions with m/z ratios of 319 (20-HETE) and 327 (d8-20-HETE) were isolated, fragmented, and the specific product ions at m/z of 245 and 253 generated from 20-HETE and the internal standard were monitored. Analyst software (Version 1.1; Applied Biosystems/MDS Sciex) was used for peak integration, determination of the calibration curve, and calculation of the concentration of 20-HETE in the samples.

Immunohistochemical Localization of CYP4A Protein After t-MCAO

The brains of the rats were removed 24 hours after t-MCAO, fixed in formalin, embedded in paraffin and cut into 3-μm-thick sections. After deparaffinized, the sections were incubated overnight with a 1:100 dilution of an anti-rat CYP4A (Affinity Bioreagents, Inc; Golden, Colo) followed by a biotinylated goat anti-rabbit IgG (10 μg/mL, Vector Laboratories, Inc; Burlingame, Calif). The sections were immersed in a methanol-hydrogen peroxide solution to quench endogenous peroxidase activity and then treated with an avidin-biotinylated peroxidase complex (Vector Laboratories, Inc). Peroxidase activity was visualized with an AEC chromagen kit (Sigma Immunochemicals; St Louis, Mo) and the sections were counterstained with hematoxylin. Control sections were incubated with nonimmune rabbit serum followed by exposure to a secondary antibody.

Primate Stroke Model

A thromboembolic stroke was induced in adult male Cynomolgus monkeys (n = 21; China National Scientific Instruments & Materials Import/Export Co; Shenzhen, China) as previously described.10 Monkeys weighing between 3.0 to 5.0 kg were anesthetized with pentobarbital (10 mg/kg, IV) and medetomidine (50 μg/kg, IM). Catheters were implanted in the carotid artery and femoral vein. After a 3-day recovery period, a blood sample was collected and

| Physiological Parameters Before and 2 Hours After MCAO |
|----------------|----------------|----------------|----------------|----------------|
|                | MAP, mm Hg     | Glucose, mg/dL | pH             | pO2, mm Hg     |
| Vehicle        | Before         | 85 ± 4         | 116 ± 5        | 7.50 ± 0.02    | 134 ± 8       | 35.4 ± 1.4 |
|                | 2 h            | 93 ± 6         | 119 ± 3        | 7.54 ± 0.01    | 160 ± 7       | 28.6 ± 1.3 |
| TS-011         | 0.001 mg/kg per hour | Before         | 86 ± 2         | 122 ± 6        | 7.53 ± 0.02    | 150 ± 8       | 33.2 ± 1.5 |
|                | 2 h            | 96 ± 4         | 127 ± 4        | 7.58 ± 0.03    | 155 ± 8       | 28.4 ± 1.7 |
|                | 0.01 mg/kg per hour | Before         | 89 ± 3         | 119 ± 5        | 7.52 ± 0.02    | 151 ± 8       | 33.4 ± 1.5 |
|                | 2 h            | 107 ± 4        | 125 ± 6        | 7.54 ± 0.01    | 167 ± 9       | 29.5 ± 1.5 |
|                | 0.1 mg/kg per hour | Before         | 84 ± 4         | 117 ± 6        | 7.52 ± 0.01    | 159 ± 8       | 32.0 ± 1.2 |
|                | 2 h            | 105 ± 3        | 118 ± 5        | 7.54 ± 0.01    | 146 ± 9       | 27.9 ± 1.1 |
|                | 1.0 mg/kg per hour | Before         | 98 ± 3         | 115 ± 5        | 7.50 ± 0.01    | 155 ± 9       | 35.5 ± 1.3 |
|                | 2 h            | 101 ± 6        | 124 ± 5        | 7.55 ± 0.02    | 170 ± 8       | 29.2 ± 1.8 |

MAP indicates mean arterial pressure.

Figure 2. Plasma levels of 20-HETE measured at various times after t-MCAO (n = 5 to 10 rats per group). TS-011 (0.01 and 0.1 mg/kg per hour) or vehicle were infused for 1 hour immediately after the initiation of reperfusion. *P < 0.05 versus the vehicle-treated group.
allowed to clot for 3 hours. The monkeys were anesthetized with sevoflurane, and a 10-cm-long clot was injected into the internal carotid artery. The animals were observed for neurological deficits before and 1, 2, 3, 5, 7 and 24 hours after embolization using a modified Japanese Stroke Scale on a 0 to 100 point scale with 28 points assigned to consciousness, 22 points related to the sensory system, 32 points assigned to motor function and 18 points related to skeletal muscle coordination.

Statistical Analysis
Mean values±SEM are presented. The significance of differences in mean values between treatment groups was evaluated using a 1-way analysis of variance followed by a Holm-Sidak, post hoc test. An unpaired Student t test was used when values from only 2 groups were compared. A Wilcoxon rank-sum test was used to determine the significance of differences in rota-rod and beam-walking test with Bonferroni correction. A probability value of <0.05 was considered to be significant.

Results

Effect of TS-011 on Infarct Volume in t-MCAO Rats
The effects of TS-011 given immediately after reperfusion on infarct volume are presented in Figure 1A. TS-011 (0.01 to 1.0 mg/kg per hour) significantly reduced infarct volume by about 40%. TS-011 had no effect on any of the other physiological parameters measured in these animals (Table).

In other experiments, TS-011 (0.1 mg/kg per hour) significantly reduced infarct volume when given 1, 2 and 4 hours after t-MCAO (Figure 1B). It had no significant effect when it was given 7 hours after MCAO (data not shown).

Effect of TS-011 on Plasma Levels of 20-HETE After t-MCAO in Rats
Baseline levels of 20-HETE averaged 520.0±41.4 pg/mL and 517.9±23.4 pg/mL, respectively in normal and sham-operated rats (Figure 2). Plasma levels of 20-HETE rose by 149% and 129%, 4 and 7 hours after t-MCAO. Plasma levels of 20-HETE were significantly reduced 4 and 7 hours after t-MCAO in rats treated with 0.1 or 1.0 mg/kg per hour TS-011.

Effect of TS-011 on Neurological Deficits in MCAO Rats
Control animals exhibited very severe sensory neurological deficits, 1 to 3 days after MCAO with and average

![Figure 3. Effect of TS-011 on the development of neurological deficits at various times after t-MCAO in rats. A, Composite sensory neurological deficits score on 8 tests. Maximum deficit score is 16. B, Time spent on a rota-rod. C, Time to cross a balance beam. *P<0.0125 versus the vehicle-treated group. D, Infarct volume measured at 7 days after t-MCAO in the same rats. *P<0.05 versus the vehicle-treated group.](downloaded from http://stroke.ahajournals.org/ by guest on April 13, 2017)
score of 14 of a possible 16 on the 8 tests. TS-011 (1.0 mg/kg per hour) given for 1 hour immediately after induction of MCAO followed by bolus injections (0.3 mg/kg) once a day for 7 days reduced the sensory deficit score by about 30% (Figure 3A). TS-011 also improved motor function. It increased the time spent on the rota-rod by 3-fold (Figure 3B) and shortened the time needed to cross a balance beam by 5-fold (Figure 3C). Total infarct volume was reduced by TS-011 by about 35% at 7 days after MCAO (Figure 3D).


Figure 5. A, Effect of TS-011 and t-PA given separately and in combination on the total neurological deficit score in monkey thromboembolic stroke model. TS-011 was given as bolus injection at a dose of 0.3 mg/kg followed by a continuous infusion at a dose of 1.0 mg/kg per hour for 24 hours after embolization. t-PA (300 000 U/kg) was given 1 hour after embolization as a 10% bolus dose followed by a continuous infusion for 2 hours. B, Effect of TS-011 and t-PA given separately and in combination on infarct size. *P < 0.05 versus the vehicle-treated group.
Expression of CYP4A Protein in t-MCAO Rats
The expression of CYP4A protein increased 24 hours after t-MCAO in endothelial cells of cerebral arteries and in cortical neurons found in the penumbral region (Figure 4A and 4B). No staining was seen in neurons or the vascular endothelium in the brains of control animal or from the contralateral nonischemic hemisphere of rats subjected to t-MCAO.

Effect of TS-011 on Neurological Deficit After Thromboembolic Stroke in Monkeys
TS-011 significantly reduced the neurological deficit score 24 hours after embolization (Figure 5A). This was associated with infarct sizes of 25±7% versus 19±4% of the affected hemisphere in animals treated with vehicle versus TS-011 (Figure 5B). However, because of the small sample size, this difference was not significant.

In other experiments, t-PA (300 000 IU/kg) given 1 hour after embolization had no significant effect at reducing the neurological deficit score in the monkeys. Combined administration of TS-011 and t-PA significantly reduced the neurological deficit score (Figure 5A). Total infarct sizes averaged 30±6% of the affected hemisphere in monkeys treated with t-PA versus 16±7% in those treated with TS-011 and t-PA.

Discussion
The present study examined the contribution of 20-HETE to the development of cerebral injury in a t-MCAO model of ischemic stroke in rats and in a thromboembolic model of stroke in monkeys. The results indicate that plasma levels of 20-HETE increased and that blockade of the synthesis of 20-HETE with TS-011 reduced infarct size and improved indices of sensory and motor function after t-MCAO in rats. Similarly, administration of TS-011 alone or in combination with t-PA significantly improved the neurological deficit score in a monkey model of thromboembolic stroke and tended to reduce infarct size. These studies support a role for 20-HETE in the pathogenesis of brain injury after ischemic stroke.

A number of therapeutic approaches reduce infarct volume after cerebral ischemia in various animal models. These agents roughly divide into agents that reduce oxygen consumption, increase CBF, scavenge free radicals, prevent elevations in intracellular Ca2+ and the activation of signal transduction cascades or compounds that block the release or actions of vasoconstrictor and chemotoxic factors.20–26 The magnitude of the reduction of infarct volume in rats treated with TS-011 in the present study is equal to, or greater than, that seen in these previous studies. One therapeutic advantage of the treatment of ischemic stroke with TS-011 is that the therapeutic window is rather broad (up to 4 hours postreperfusion). Most of the other treatments only have beneficial effects when given before induction of the stroke or shortly thereafter.

The mechanism by which 20-HETE inhibitors reduce infarct volume after cerebral ischemia and reperfusion remains to be determined. Both polymorphonuclear leukocytes and cerebral arteries produce 20-HETE,3,27 and there is massive release of AA during cerebral ischemia.3 Thus, it is likely that the local production of 20-HETE in cerebral arteries increase during ischemic stroke. We also obtained evidence for upregulation of the expression of CYP4A protein in neurons and vascular endothelial cells 24 hours after ischemia reperfusion injury in rats. The present finding that the plasma levels of 20-HETE increase 4 to 7 hours after t-MCAO in rats further supports this possibility. 20-HETE is a potent vasoconstrictor of cerebral arteries, and inhibitors of 20-HETE attenuate the development of delayed and acute vasospasm in animal models.10,13,14 Thus, it is possible that TS-011 reduces infarct size by improving CBF and oxygen delivery to the penumbra either during or after t-MCAO. However, 20-HETE also inhibits Na+, K+-ATPase activity4 and activates a number of cell signaling pathways. Thus, TS-011 may reduce cell swelling, rupture of intracellular organelles and the apoptosis of neurons after ischemia and reperfusion of the brain. TS-011 may also prevent depolarization of neurons and the release of excitatory neurotransmitters or vasoactive and chemotoxic factors that have been implicated in the death of neurons after ischemic injury. Thus, additional studies are needed to determine whether 20-HETE synthesis inhibitors and antagonists reduce infarct size by improving blood flow or via a direct effect on the survival of neurons after ischemia and reperfusion injury.

Administration of t-PA did not improve the neurological outcome or reduce infarct size in the thromboembolic model of stroke in monkeys (Figure 5). The reason for this is that in some of the monkeys t-PA produced intracerebral hemorrhage (ICH) and increased rather than decreased infarct area especially in posterior region of the brain. In contrast, the neurological deficit score was significantly improved and infarct size tended to be reduced in animals that received combined therapy with TS-011 and t-PA. These results suggest that TS-011 may decrease the consequence of cerebral vasospasm associated with ICH in animals treated with t-PA. This interpretation is consistent with the results of recent studies of SAH and ICH in rats.9,10,13,15

In conclusion, the results of the present study indicate that inhibition of the formation of 20-HETE reduces infarct size and improves neurologic outcome in animal models of ischemic stroke. This suggests that TS-011 might be useful for the treatment of ischemic stroke.

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