JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964

Beneficial Effects of a new 20-HETE Synthesis Inhibitor, TS-011, on Hemorrhagic and Ischemic Stroke

Noriyuki Miyata, Takayuki Seki, Yu Tanaka, Tomohiro Omura, Kazuo Taniguchi, Mariko Doi, Kagumi Bandou, Shunichi Kametani, Masakazu Sato, Shigeru Okuyama, Liana Cambj-Sapunar, David R. Harder and Richard J. Roman

Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., Saitama, Japan (N.M., T.S., Y.T., T.O., K.T., M.D., K.B., S.K., M.S., S.O.); Department of Physiology, Medical College of Wisconsin, Milwaukee, WI 53226, U.S.A (L.C-S., R.J.R); Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, WI 53226, U.S.A (D.R.H.)

a) Running title: Potent and Selective Inhibitor of 20-HETE Synthesis

b) Address for correspondence: Noriyuki Miyata, Ph.D.
Medicinal Pharmacology Laboratory
Medicinal Research Laboratories,
Taisho Pharmaceutical Co., Ltd.
1-403 Yoshino-cho, Kita-ku,
Saitama-city,
Saitama 331-9530,
Japan
Phone: +81-48-669-3089
FAX: +81-48-652-7254
E-mail: noriyuki.miyata@po.rd.taisho.co.jp

c) The number of text pages: 22
The number of tables: 4
The number of figures: 7
The number of references: 36
The number of words in the Abstract: 250
The number of words in Introduction: 704
The number of words in Discussion: 1170

d) ABBREVIATIONS: AA, arachidonic acid, ABT, 1-aminobenzotriazol; CSF, cerebrospinal fluid; 20-HETE, 20-hydroxyeicosatetraenoic acid; SAH, subarachnoid hemorrhage; TS-011, *N*-(3-Chloro-4-morpholin-4-yl) phenyl- *N'*-hydroxyimido formamide; t-PA, tissue plasminogen activator; WIT0002, 20-hydroxy-6(Z), 15(Z)-dienoic acid; 17-ODYA, 17-octadecynoic acid; DDMS, dibromododecinoic acid, MAP, mean arterial pressure; ICH, intracerebral hemorrhage; HET0016, N-hydroxy-N'-(4-butyl-2-methylphenyl)- formamidine; rCBF, regional cerebral blood flow; CYP, cytochrome P450; FRBC, fluorescently-labeled red blood cells; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; PAF, platelet activating factor; 10-SUYS, sodium 10-undecynyl sulfate; TTC, 2,3,5-triphenyltetrazolium chloride;

e**) Section options:** Cardiovascular

ABSTRACT

The present study characterized the effects of TS-011 (*N*-(3-Chloro-4-morpholin-4-yl) phenyl-*N'*-hydroxyimido formamide), a new selective inhibitor of the synthesis of 20-hydroxyeicosatetrenoic acid (20-HETE), on the metabolism of arachidonic acid by human and rat renal microsomes and the inhibitory effects of this compound on hepatic cytochrome P450 (CYP) enzymes involved in drug metabolism. The effects of TS-011 on the fall in cerebral blood flow following subarachnoid hemorrhage (SAH) and in reducing infarct size in ischemic stroke models were also examined since 20-HETE may contribute to the development of cerebral vasospasm. TS-011 inhibited the synthesis of 20-HETE by human renal microsomes and recombinant CYP4A11 and 4F2, 4F3A and 4F3B enzymes with IC_{50}s around 10-50 nM. It had no effect on the activities of CYP1A, 2C9, 2C19, 2D6 or 3A4 enzymes. TS-011 inhibited the synthesis of 20-HETE by rat renal microsomes with an IC_{50} of 9.19 nM, and it had no effect on epoxygenase activity at a concentration of 100 μM. TS-011 (0.01-1 mg/kg, *i.v.*) reversed the fall in cerebral blood flow and the increase in 20-HETE levels in the cerebrospinal fluid of rats after SAH. TS-011 also reduced the infarct volume by 35% following transient ischemic stroke and in intracerebral hemorrhage in rats. Injection of 20-HETE (8 or 12 mg/kg) into the carotid artery produced an infarct similar to that seen in the ischemic stroke model. These studies indicate that blockade of the synthesis of 20-HETE with TS-011 opposes cerebral vasospasm following SAH and reduces infarct size in ischemic models of stroke.

Introduction

Each year, 750,000 strokes occur in the United States. Approximately, 80% of the strokes are ischemic and 20% are hemorrhagic. Hemmorrhagic stroke commonly occurs after rupture of aneurysms or head trauma. There is an initial period of cerebral ischemia lasting several hours due to a rise in cerebral spinal fluid (CSF) pressure and acute cerebral vasospasm. Half of the patients that survive later develop delayed vasospasm. The mortality in the first month hovers around 50%. The majority of deaths (>60%) occur during the first 2 days and is associated with ischemic brain injury (Broderick et al., 1994). The factors that contribute to the acute fall in cerebral blood flow following subarachnoid hemorrhage (SAH) remain uncertain.

Ischemic strokes are caused by blockage of cerebral arteries. In the ischemic neuronal tissue there is depletion of ATP, cellular acidification (Sarvary et al., 1994) and a rise in intracellular Na^{+} and Ca^{++} concentrations (Kristian et al., 1998). With prolonged ischemia (0.5 to 2 hrs), intracellular organelles rupture, release of proteolytic enzymes and neurons die. There is also release of vasoconstrictors and neurotransmitters by ischemic neurons (Guyot et al., 2001; Tseng et al., 1999; Werling et al., 1993). The release of the neurotransmitters contribute to brain injury by increasing metabolic demand, while the vasoconstrictors oppose the recruitment of collateral flow. The size of the infarct can be minimized if blood flow is restored within 2 hrs after the onset of the stroke or if the metabolic demand of the tissue is reduced.

Current treatments for hemorrhagic stroke focuses on the repair of aneurysms and reductions intracranial pressure. Osmotic agents and steroids have also been tried to reduce cerebral edema and pressure. However, these treatments have proven to be largely ineffective.

Recent studies have indicated that 20-HETE may contribute to the development of vasospasm following SAH. In this regard, 20-HETE is a potent constrictor of cerebral arteries (Gebremedhin et al., 1998; 2000) and the levels of 20-HETE increase in CSF following SAH (Kehl et al., 2002; Cambj-Sapunar et al., 2003). Inhibition of the synthesis of 20-HETE, with 17-ODYA or HET0016, or blockade of its vasoconstrictor actions with 20-hydroxy-6(Z), 15(Z)-dienoic acid (WIT0002) prevents the acute fall in cerebral flow following SAH (Kehl et al., 2002; Yu et al., 2004). There is also evidence that blockade of the synthesis of 20-HETE may also reverse delayed vasospasm in dogs (Hacein-Bey et al., 2004a).

The only approved treatment for ischemic stroke is the administration of tissue plasminogen activator (t-PA) to dissolve clots and restore cerebral blood flow (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). There are no approved

agents to increase collateral flow following ischemic stroke. Since the levels of arachidonic acid (AA) in the CSF was high during ischemic stroke (Pilitsis et al., 2003), it is possible that the production of 20-HETE in cerebral arteries increases and that this might contribute to brain injury by opposing the recruitment of collateral flow. 20-HETE also activates several signal transduction pathways (Sun et al., 2000; Muthalif et al., 2000; Randriambozvonjy et al., 2003) involved in apoptosis and cell death. Thus, blockade of the formation of 20-HETE may reduce infarct size following ischemic stroke similar to its ability to restore cerebral blood flow following SAH. However, little progress has been in developing 20-HETE inhibitors for the treatment of stroke. The current available compounds, dibromododecinoic acid (DDMS), 17-octadecynoic acid (17-ODYA), 1-aminobenzotriazol (ABT) or sodium 10-undecynyl sulfate (10-SUYS), are not very potent and/or selective inhibitors for synthesis of 20-HETE (Wang et al., 1998; Su et al., 1998; Miyata et al., 2001; Xu et al., 2002) and 20-HETE antagonists are ineffective because they do not cross the blood brain barrier. HET0016 is the most selective inhibitor of the synthesis of 20-HETE (Miyata et al., 2001). However, this compound is not very soluble and has a limited half-life (Nakamura et al., 2003). Thus, new and more specific inhibitors are needed to evaluate the role of 20-HETE in various models of stroke. The present study characterize the effects of a new derivative of hydroxyformamidine, TS-011 (*N*-(3-Chloro-4-morpholin-4-yl) phenyl- *N'*-hydroxyimido formamide (Fig. 1) to inhibit the formation of 20-HETE, prevent the fall in CBF following SAH and in reducing infarct size in a rat model of ischemic stroke.

Methods

General. Experiments were performed on 5 week old Spontaneously Hypertensive rats (SHR) and 8 week old IGS rats purchased from Charles River Laboratories (Atsugi, Japan), 9-11 week old male Sprague Dawley rats from Harlan Sprague Dawley Laboratories (Indianapolis, IN) and 9-11 week old male Wistar rats SLC (Shizuoka, Japan). The rats were housed in approved animal care facilities either at the Medical College of Wisconsin or Taisho Pharmaceutical Co and had free access to food and water throughout the study. All protocols were reviewed by the Animal Care Committee of Taisho Pharmaceutical Co., Ltd. or the Medical College of Wisconsin and they conformed to the recommendations for the care of laboratory animals established by the National Institutes of Health and the Japanese Experimental Animal

Research Association (1987).

Characterization of TS-011 as a selective inhibitor of the formation of 20-HETE by CYP4A and 4F Enzymes. The effects of TS-011 on the formation of EETs and 20-HETE by human and rat renal microsomes were studied since they are rich mixed source of the CYP isoforms (CYP2C, CYP2J, CYP2E, CYP4A, CYP4F and CYP4B) known to metabolize AA. The structure of TS-011 is presented in Fig. 1 and was synthesized in the Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). Human renal microsomes were purchased from Human Cell Culture Center (Laurel, MD, U.S.A.). Microsomes were also prepared from the kidneys of 6 male SHR as follows. The rats were anesthetized with pentobarbital (50 mg/kg, *i.p.*), the kidneys were removed and renal cortex was homogenized in a 20 mM HEPES (pH 7.4) buffer containing; 1 mM ethylene-diaminetetraacetic acid (EDTA), 100 μM *p*-(amidinophenyl)-methanesulfonyl fluoride, and 250 mM sucrose. The homogenate was centrifuged at 600 g for 10 min and 16,000 g for 30 min. The supernatant was collected and centrifuged at 200,000 g for 30 min. The resulting pellet was suspended in 50 mM MOPS buffer.

Microsomes (200 μg/ml protein) were preincubated with various concentrations of TS-011 (10^{-12} to 10^{-4} M for 10 min at 37^{o} C in 100-200 μl of a 50 mM MOPS buffer (pH 7.4) containing 5 mM $MgCl_2$ and 1 mM EDTA. [^{3}H]-Arachidonic acid (2 μCi/ml, 0.01 μM) and NADPH (1 mM) were added and the reactions were incubated for 20 min for EET or 1 hr for 20-HETE for the rat microsomes and for 6 hrs for the human microsomes. The reactions were stopped by the addition of formic acid (pH 3.5). Acetonitrile was added to adjust the final concentration to 50% and the samples were separated by HPLC on a 150×4.6 mm Bio-sil C18HL90-5S column (Absorbosphere C18, Alltech Associates, Inc., Deerfield, IL, U.S.A.) at a flow rate of 1.0 ml/min using a linear elution gradient ranging from acetonitrile:water: acetic acid (50:50:0.1 vol/vol/vol) to acetonitrile:acetic acid (100:0.1) over 30 min. The metabolites formed were monitored using a radioactive flow detector (Ramona Star, Raytest GmbH, Straubenhardt, Germany). The identity of each metabolite was confirmed by comigration with known standards.

The ability of TS-011 to inhibit the formation of 20-HETE by the major CYP isoforms known to produce 20-HETE in man were also examined. Microsomes prepared from baculovirus infected insect cells that express human recombinant CYP4A11, 4F2, 4F3A and 4F3B enzymes (Gentest Corp, Woburn, MA, U.S.A.) were preincubated for 5 min at 37^{o} C with various concentrations of TS-011 (10^{-9}-10^{-4} M) or vehicle in 1 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$. [^{14}C]-arachidonic acid (5 μCi/ml, 10 μM). After the

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

preincubation period 1 mM NADPH was added and the reactions were incubated for 20 min at 37 ^{o}C. The reaction was terminated by acidification with formic acid, the metabolites were extracted with ethyl acetate and the metabolites were separated and monitored by HPLC as described above.

Effects of TS-011 on CYP-1A2, CYP-2C9, CYP-2C19, CYP-2D6, and CYP-3A4 activities. The selectivity of TS-011 as an inhibitor of CYP isoforms involved in the formation of 20-HETE was further tested by studying the ability of this compound to inhibit the activity of the major human hepatic CYP enzymes involved in drug metabolism. In these experiments, 0.5-3 pmoles of microsomes prepared from baculovirus infected insect cells expressing recombinant human CYP-1A2, CYP-2C9, CYP-2C19, CYP-2D6, CYP-3A4 enzymes (Gentest Corp, Woburn, MA) were incubated for 15-45 min with an appropriate fluorescent substrate, 0.08 or 1.3 mM NADPH and TS-011 (10^{-8}-10^{-4} M) or vehicle. The substrates used were 0.5 pmol (enzyme) and 5 μM 3-cyano-7-ethoxycoumarin for CYP-1A2; 75 μM 7-methoxy-4-trifluoromethyl-coumarin for CYP-2C9; 1.5 μM 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7methoxy-4-methylcoumarin for CYP2D6; 25 μM 3-cyano-7-ethoxycoumari for CYP-2C19 and 40 μM 7-benzyl-oxyquinoline for CYP-3A4. The formation of the fluorescent metabolites were monitored using the following excitation and emission wavelengths: CYP-1A2: 405 nm and 460 nm; CYP-2C9: 405 nm and 535 nm; CYP-2C19: 405 nm and 460 nm; CYP-2D6: 390 nm and 460 nm; CYP-3A4: 405 nm and 535 nm, respectively. The experiments were repeated 6 times for each enzyme and IC_{50} values for TS-011 on each enzyme were determined according to the method of Crespi et al. (1997).

Characterization of the effects of TS-011 on various receptors and enzymes. These binding and enzyme assays were performed as a contract study by CEREP (Celle l'Evescault, France). A list of the assays employed is provided in Table 2. All experiments were performed at least in duplicate.

Effects of pretreatment of rats with TS-011 on the acute fall in cerebral blood flow after SAH: Prevention protocol. These experiments were performed on 9-12 weeks old male Sprague-Dawley rats. The rats were anesthetized with ketamine (20 mg/kg *i.m.*) and thiobutabarbital (Inactin; 50 mg/kg *i.p.*) and surgically prepared for induction of SAH and measurement of CBF through an intact cranial window as previously described (Kehl et al., 2002; Cambj-Sapunar et al., 2003). The head of the rat was placed in a stereotaxic apparatus and the atlantooccipital membrane was exposed by separating the muscle layers at the base of the

skull. A cannula was placed in the cisterna magna for injection of blood and a pulled PE-10 cannula was placed under the dura for measurement of intracranial pressure (ICP). CBF was measured using laser-Doppler flowmetry after thinning the bone on the parietal cortex. A cannula was placed in the trachea and the rats were artificially ventilated with a mixture of 30 % O_2 in N_2. End-tidal P_{CO2} was maintained at 35 mmHg. Cannulas were also placed in the femoral artery and vein for infusion of drugs and measurement of mean arterial blood pressure (MAP). Body temperature was maintained at 37 ^oC and the rats received an *i.v.* infusion of 0.9 % NaCl solution containing 1 % bovine serum albumin at a rate of 3 ml/hour to replace fluid losses.

After surgery and a 30 min equilibration period, baseline CBF and ICP were measured. The rats then received an *i.v.* injection of vehicle (N=17) (11 % sulfobutylether-beta-cyclodextrin) or TS-011 at doses of 0.001 (N=5), 0.01 (N=6) or 0.1 (N=12) mg/kg and CBF and ICP were measured for an additional 30 min. After the control value of CBF was established, 0.3 ml of unheparinized arterial blood was infused into the cisterna magna at a rate of 30 μl/min over a 10 min period. Control animals received an equal volume of aCSF. CBF and ICP were recorded at 10, 20, 30, 60, 90, 120 min after induction of SAH. At the end of most experiments, CSF (~100 μl) was withdrawn from the cannula placed in the cisterna magna, transferred to glass vials and stored at -80^o C. 20-HETE concentration in these samples was measured with a fluorescent HPLC assay as previously described (Maier et al., 2000).

Effects of TS-011 on the fall in CBF following SAH: Reversal protocol. Experiments were performed to determine whether TS-011 could not only prevent, but also reverse, the fall in CBF following SAH. CBF was measured with laser-Doppler flowmetry a control period and for 30 min after induction of SAH. The rats then received an intravenous injection of TS-011 (0.1 mg/kg, N=7) or an equal volume of vehicle (N=7) and CBF was followed for an additional 120 min.

Effects of TS-011 on infarct size following transient occlusion of the MCA of rats. Male Wistar rats were anesthetized with 1 % halothane. A catheter was placed in the femoral artery for blood sampling and measurement of mean arterial pressure (MAP). Transient MCAO was produced by the intraluminal suture method described in detail elsewhere (Nagasawa and Kogure 1989). The junction of the right common carotid artery, external cerebral artery, and internal cerebral artery were exposed. An 18-mm length of nylon suture (4-0) (Nitcho Kogyo Co., Ltd.) coated with silicon (Xantopren VL plus, Hraeus Kulzer Dental Products Division) was introduced into the internal cerebral artery and advanced 13 mm to the origin of the right MCA. The neck

wounds were closed, anesthesia was withdrawn and successful occlusion was of the MCAO tested by the appearance of left side hemiparesis. Rats that did not demonstrate hemiparesis were excluded from analysis. After 60 minutes of occlusion of the MCAO, the rats were reanesthetized with halothane and the suture blocking the MCA were withdrawn allowing reperfusion. TS-011, at doses of 0.001, 0.01, 0.1, or 1.0 mg/kg (*i.v.*), or vehicle (10 % hydroxypropyl-β cyclodextrin) was administered to approximately 20 rats per group just prior to reperfusion of the MCA. Thirty minutes after reperfusion of the MCA, the rats were allowed to awake from anesthesia. Twenty four hours later the rats were anesthetized with diethyl ether, decapitated and the brains removed and cut into seven 2-mm thick coronal sections (from +4 mm to -8 mm bregma). The sections were immersed in a 2 % (w/v) 2, 3, 5-triphenyltetrazolium chloride (TTC) solution at 37 °C for 30 min, and subsequently fixed with 10 % (v/v) buffered formalin solution. Cortical and sub-cortical infarct areas were measured using NIH-image analysis software.

Effect of carotid artery infusion of 20-HETE on infarct volume in rats. Experiments were performed to determine whether an increase in the levels of 20-HETE in the cerebral circulation could produce a cerebral infarct similar to that seen following transient occlusion of the MCA. The experiments were performed according to the method by Shirakura et al. (1992). Rats were anesthetized with 1% halothane. The right carotid artery was exposed via a midline incision and a non-occluding catheter was introduced. 20-HETE (8 or 12 mg/kg) dissolved in 0.1 ml of 0.1 M Na_2CO_3 solution or an equal volume of vehicle was infused over a 15 second period.
The dose of 20-HETE was decided from the results of preliminary studies to obtain the reproducible infarct volume and neurologic deficits. The catheter was then removed the neck closed and the animal was allowed to recover from surgery. Twelve hours later, circling behavior, beam walking, and forelimb extension tests were performed to access the degree of motor dysfunction. Higher neurologic deficit score denotes more severely impaired function. The rats were then anesthetized with diethyl ether and the brains collected and stained with TTC to access infarct volume.

Effects of TS-011 on infarct size following collagenase-induced intracerebral hemorrhage. We also examined the effects of TS-011 on infarct size in a collagenase-induced model of intracerebral hemorrhage (Del Bigio et al., 1996). Briefly, 8 week old Sprague Dawley rats purchased from Charles River Laboratories (Atsugi, Japan) were anesthetized with thiopental (30 mg/kg, *i.p.*) and the head place in a stereotaxic apparatus. The small hole was drilled in the skull (3 mm left, 0.2 mm posterior to bregma), and a 30-gauge needle was inserted 6 mm below the surface

of the skull into the caudate nucleus. 0.7 μL of a solution of collagenase (200 Units/ml in saline) was infused into the brain over a 5 min period. The needle was withdrawn, the hole in the skull sealed with bone wax and the skin incision was closed. TS-011, at doses of 0.001, 0.01 and 0.1 mg/kg (*i.v.*), or vehicle (10 % hydroxypropyl-β cyclodextrin) was administered immediately after collagenase injection. Twenty four hours later, circling behavior, beam walking, and forelimb extension tests were performed to access the degree of motor dysfunction according to the method of Peeling et al. (1988). Higher neurologic deficit score denotes more severely impaired function. The brain was removed to access infarct volume as described above.

Statistical analysis. Mean values ± SE are presented. Significance of differences in mean values within and between groups was examined by a two-way ANOVA for repeated measures followed using a Dunnett's test for planned comparisons. A P value < 0.05 was considered to be significant.

RESULTS

Characterization of TS-011 as a selective inhibitor of the formation of 20-HETE by CYP4A and CYP4F enzymes. Microsomes prepared from human kidney only produced 20-HETE when incubated with AA. TS-011 inhibited the formation of 20-HETE by human renal microsomes with an IC_{50} (95% confidence interval) of 8.42 nM (4.28-16.58nM, Fig. 2a). Microsomes prepared from the kidneys of SHR produced both 20-HETE and 11, 12-EET. TS-011 selectively inhibited the formation of 20-HETE formation by rat renal microsomes with an IC_{50} of 9.19 nM (3.93-21.50 nM, Fig. 2b). It had no effect on the formation of EETs even at a concentration of 100 μM (Fig. 2b).

The effects of TS-011 on the CYP isoforms (CYP4F2, 4F3A , 4F3B and 4A11) reported to produce 20-HETE in man are presented in Fig. 2c-f. Microsomes prepared from baculovirus infected insect cells that express these isoforms only produced 20-HETE when incubated with AA. TS-011 inhibited the production of 20-HETE by these isoforms in a concentration-dependent manner and IC_{50} values (95% confidence interval) averaged 30.4 nM (28.0-33.0 nM) for CYP4F2, 42.6 nM (38.2-47.5 nM) for CYP4F3A, 43.0 nM (33.8-54.6 nM) for 4F3B and 188 nM (116-303 nM) for 4FA11 (Fig. 2 c-f).

Effects of TS-011 on CYP-1A2, CYP-2C9, CYP-2C19, CYP-2D6, and CYP-3A4 activities TS-011 had little effect on the activities of these human hepatic CYP isoforms involved in drug

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

metabolism. The IC_{50} for CYP1A2-catalyzed substrate oxidation was 60.8 μM. The IC_{50} values for CYP2C9-, CYP2C19-, CYP2D6 and CYP3A4-catalyzed substrate oxidation reactions were all >100 μM (Table 1).

Characterization of the effects of TS-011 on other receptors and enzymes. The results of these experiments are summarized in Table 2. TS-011 was screened for binding to a broad range of receptors and for inhibitory effects on activity of many enzymes. TS-011 exhibited little or no affinity for any of the receptors tested. It also had no significant effect on the activity of any of the enzymes tested at a concentration 10^{-6} M.

Effect of TS-011 on CBF and 20-HETE levels in CSF after SAH. The effects of pretreatment of the rats with various doses (0.001 to 0.1 mg/kg) of TS-011 on the changes in CBF following SAH in rats is presented in Fig. 3a. Injection of aCSF into the cisterna magna had no effect of CBF. In contrast, CBF fell by 60% in vehicle treated rats during the injection of blood into CSF (Fig. 3a). This corresponded with a rise in ICP from about 10 to 60 mm Hg (Fig. 3b) while MAP was unaltered. Within 10 min after the infusion of blood into the CSF, ICP returned to 30 mmHg and CBF rose to 70% of control. It remained at this level for the 2 hr course of the experiment. TS-011 had no effect on baseline CBF and it did not affect the initial fall in CBF or rise in ICP seen after induction of SAH. However, pretreatment of rats with TS-011 at doses of 0.01 and 0.1 mg/kg prevented the sustained fall in CBF following SAH. In these animals, CBF rapidly returned to values not significantly different from control after induction of SAH. CBF also recovered in the rats pretreated with the lowest dose (0.001 mg/kg) TS-011 but it took longer to fully recover.

A comparison of the effects of TS-011 (0.1 mg/kg) on CBF when given 30 min before, or 30 min after induction of SAH is presented in Fig. 4. TS-011 returned CBF completely to control within 2 hrs when given therapeutically, 30 min after induction of SAH when rCBF was already reduced.

The effects of TS-011 on the changes in 20-HETE levels in CSF seen following induction of SAH are presented in Fig. 5. 20-HETE levels in CSF were relatively low and average <100 ng/ml in sham operated control animals in which we did not prepare a thinned cranial window for measurement of CBF. The baseline levels of 20-HETE in CSF were higher in rats prepared with a thinned cranial window. 20-HETE levels increased markedly 2 hrs after induction of SAH. In contrast, the levels of 20-HETE remained unaltered in rats that received an injection of aCSF in the CSF. Administration of TS-011 given either 30 min before or 30 min after induction of

SAH had a similar effect to reduce the levels of 20-HETE in CSF following SAH. Thus, the results from these two groups were pooled and presented together in Fig. 5.

Effects of TS-011 on infarct size following transient occlusion of the MCA in rats. The results of these experiments are summarized in Fig. 6. Total, cortical, and sub-cortical infarct volumes in the vehicle-treated rats 24 hours after transient occlusion of the MCA averaged 194 ± 19, 136 ± 15, and 57.1 ± 4.0 mm^3, respectively (n=22). Total and cortical infarct volumes were significantly reduced in rats given 0.01 and 0.1 mg/kg TS-011. Sub-cortical infarct volume was also significantly reduced in rats given 1 mg/kg TS-011.

Effect of intra-arterial infusion of 20-HETE on infarct volume in rats. The results of the experiments to determine whether an increase in the levels of 20-HETE in the cerebral circulation could produce an ischemic infarct that resembles that seen following transient occlusion of the MCA is presented in Fig. 7. No infarct or neurological deficits were observed in control rats that received an infusion of vehicle in the carotid artery. In contrast, a large cortical infarcts and severe neurologic deficits were observed in the rats that received an infusion of 20-HETE into the carotid artery (Table 3).

Effects of TS-011 on infarct size following collagenase-induced intracerebral hemorrhage. The results of these experiments are presented in Table 4. The area of cerebral infarct in the control rats averaged 42 $\pm$ 5 mm^2 and these rats exhibited severe motor neurological deficits, including circling behavior, the inability to walk across a beam and muscle weakness in the front forepaws. TS-011 dose-dependently reduced infarct size up to 30% and reduced the degree of motor deficit.

DISCUSSION

The present study characterized the effects of TS-011 on the formation of 20-HETE by human and rat renal microsomes and recombinant CYP4A and 4F enzymes as well its potential effects to prevent cerebral vasospasm following SAH and to reduce infarct size following transient occlusion of the MCA. The results indicate that TS-011 is the most potent and selective inhibitor of the formation of 20-HETE that has yet to be identified. The IC_{50} values for inhibition of the formation of 20-HETE by rat and human renal microsomes averaged 9.19 and 8.42 nM, respectively (Figs. 2a, b). This is an order of magnitude lower concentration than the reported

IC_{50} for HET0016 (Miyata et al., 2001). TS-011 is 100 to 1000 times more potent inhibitor of the formation of 20-HETE than DDMS, ABT, 17-ODYA or 10-SUYS (Wang et al., 1998; Su et al., 1998; Miyata et al., 2001; Xu et al., 2002). TS-011 exhibited a 1000 fold selectivity for inhibition of the formation of 20-HETE versus other CYP enzymes that metabolize AA. It had no effect on the formation of EETs by rat renal microsomes even when used at a concentration of 100 μM. We demonstrated that TS-011 is a potent and specific inhibitor of all of the CYP4F and CYP4A isoforms that are known to produce of 20-HETE in man (Lasker et al., 2000) and that it had no effect on the activity of the human hepatic CYP enzymes involved in drug metabolism at concentrations up to 60 μM (Table 1). TS-011 was also screened for binding to known classes of receptors and for inhibitory effects on a broad range of enzymes. TS-011 did not bind to any receptor type strongly, and it had no significant effect on any enzyme activity at concentrations of up to 10^{-6} M. We also confirmed that 1 μM TS-011 had no effect on 20-HETE-induced vasoconstriction in isolated canine basilar artery with endothelium (Supplemental data). This result indicates that TS-011 has no direct inhibitory effect on 20-HETE-induced response. Thus, it appears that TS-011 is a potent and selective inhibitor of CYP4F and CYP4A enzymes that catalyze the formation of 20-HETE in a variety of tissues in both man and rats.

Recent studies have indicated that the levels of 20-HETE increase in the CSF of rats (Kehl et al., 2002; Cambj-Sapunar et al., 2003), dogs (Hacein-Bey et al., 2004a) and man (Hacein-Bey et al., 2004b) following SAH and inhibition of the synthesis of 20-HETE, with 17-ODYA or HET0016 (Kehl et al., 2002; Cambj-Sapunar et al., 2003), or blockade of its vasoconstrictor actions with 20-hydroxy-6(Z), 15(Z)-dienoic acid, WIT0002 (Yu et al., 2004) can prevent the acute fall in CBF. There is also evidence that blockade of the synthesis of 20-HETE can even reverse delayed vasospasm in dogs subjected to the dual hemorrhage model (Hacein-Bey et al., 2004a). These studies suggest that blockade of the synthesis and/or actions of 20-HETE may be useful in limiting cerebral ischemia and injury to the brain following SAH, head injury and other conditions associated with intracranial bleeding. Nevertheless, the currently available 20-HETE inhibitors have limited potential as therapeutics agents for the treatment of stroke and vasospasm. For example, DDMS, 17-ODYA, ABT, 10-SUYS are not very potent or selective inhibitors of the synthesis of 20-HETE (Wang et al., 1998; Su et al., 1998; Miyata et al., 2001; Xu et al., 2002). They inhibit both the synthesis of 20-HETE and EETs. DDMS, 17-ODYA and WIT0002 also avidly bind to plasma proteins and have minimal bioavailability when delivered systemically. Finally, HET0016 has a limited half-life and is poorly soluble (Nakamura et al., 2003). Thus, the

present study evaluated the therapeutic potential of TS-011 in preventing acute cerebral vasospasm following SAH. Pretreatment of rats with TS-011 significantly lowered the levels of 20-HETE in the CSF after SAH and prevented the sustained fall in CBF. TS-011 also reversed acute vasospasm and returned rCBF to control when given therapeutically, 30 min after the induction of SAH. These studies suggest that TS-011 is effective at opposing acute vasospasm and minimizing cerebral ischemia and neurologic deficits following SAH. The half life of TS-011 in rats was 10 min when TS-011 was administered intravenously and the main metabolite of TS-011 had no inhibitory effect on 20-HETE synthesis in human renal microsomes.

The present study also examined the potential of TS-011 on minimizing infarct size following transient occlusion of the MCA, since previous studies have indicated that there is release of fatty acids including AA following ischemic stroke (Pilitsis et al., 2003). The results indicate that administration of 0.01 or 0.1 mg/kg of TS-011 significantly reduced infarct volume in rats following transient occlusion of the MCA by 35%. In this regard, blockade of 20-HETE formation with TS-011 is as, or more effective than NMDA (Liu et al., 1996a), endothelin (Patel et al., 1996), leukotriene (Aspey et al., 1997), PAF (Liu et al., 1996b) and Ca channel blockers (Shino et al., 1991) in reducing infarct size in this model. Effect of TS-011 on minimizing infarct volume was stronger in cortex than sub-cortex area. Thus, it is possible that the production of 20-HETE in cerebral arteries in cortical penumbra region might increase following ischemic stroke and inhibition of 20-HETE formation by TS-011 might rescue the cortical damage. However, future investigation is necessary to clarify the exact mechanism by which TS-011 improves the infarct volume, and possible combination therapy with t-PA that is the only approved drug for ischemic stroke. In further studies, we found that injection of 20-HETE into the carotid artery of rats produced a massive cerebral infarct that resembled that seen following transient occlusion of the MCA. These studies suggest that the release of AA and elevations in 20-HETE levels in the brain compromise CBF and contribute to the cerebral injury following ischemia/reperfusion of the brain. AA is known to induce endothelial damage and edema in the brain (Shirakura et al., 1992). However, future study is necessary to clarfy the precise mechanism by which 20-HETE caused infarct volume and neurologic deficits.

Finally, the potential beneficial effects of TS-011 in reducing infarct volume in collagenase-induced model of ICH were examined. Administration of TS-011 at a dose of 0.1 mg/kg significant reduced infarct size and the degree of neurological deficit in rats following this model of ICH. The neurological deficits in this model is thought to be due the destruction of

brain tissue secondary to cerebral edema and reduced blood flow in the affected tissue (Del Bogie et al., 1996; Jenkins et al., 1989). While the mechanism of the beneficial effect of TS-011 remains to be determined, one likely possibility is that TS-011 might improve the rCBF following ICH by preventing acute vasospasm similar to its effects following SAH.

In summary, the present results indicate that TS-011 is the most potent and selective inhibitor of the synthesis of 20-HETE that has been developed to date. TS-011 prevents acute cerebral vasospasm following SAH and provides the first evidence that blockade of the synthesis of 20-HETE can reduce infarct size in rat models of ischemic stroke and ICH.

Acknowledgements

We thank K. Kitano, C. Koizumi, Y. Yasuda, M. Ohnishi, T. Hiyoshi, Y. Yoshizawa, N. Kawashima, T. Ishii, H. Amada, Y. Kobayashi-Matsunaga, Y. Nakanishi and M. Kawanishi for their technical assistance.

References

Aspey BS, Alp MS, Patel Y, and Harrison MJ (1997) Effects of combined glutamate and platelet activating factor inhibition on the outcome of focal cerebral ischaemia- and initial screening. *Metab Brain Dis* **12:**237-249.

Broederick JP, Brott TG, Duldner JE, Tomsick T, Leach A (1994) Initial and recurrent bleeding are the major causes of death following subarachnoid hemorrhage. *Stroke* **25:**1342-1347.

Cambj-Sapunar L, Yu M, Harder DR, and Roman RJ (2003) Contribution of 5-hydroxy-tryptamine1B receptors and 20-hydroxyeiscosatetraenoic acid to fall in cerebral blood flow after subarachnoid hemorrhage. *Stroke* **34:**1269-1275.

Crespi CL, Miller VP, and Penman BW (1997) Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Anal Biochem* **248:**188-190.

Del Bigio MR, Yan HJ, Buist R, and Peeling J (1996) Experimental intracerebral hemorrhage in rats. Magnetic resonance imaging and histopathological correlates. *Stroke* **27:**2312-2319.

Gebremedhin D, Lange AR, Narayanan J, Aebly MR, Jacobs ER, and Harder DR (1998) Cat cerebral arterial smooth muscle cells express cytochrome P450 4A2 enzyme and produce the vasoconstrictor 20-HETE which enhances L-type Ca^{2+}current. *J Physiol* (Lond) **507:** 771-781.

Gebremedhin D, Lange AR, Lowry TF, Taheri MR, Birks EK, Hudetz AG., Narayanan J, Falck JR, Okamoto H, Roman RJ, Nithipatikom K, Campbell WB, and Harder DR (2000) Production of 20-HETE and its role in autoregulation of cerebral blood flow. *Circ Res* **87:** 60-65.

Guyot LL, Diaz FG, O'Regan MH, McLeod S, Park H, Phillis JW (2001) Real time measurement of glutamate release from the ischemic penumbra of the rat cerebral cortex using a focal middle cerebral artery occlusion model. *Neurosci Lett* **299:**37-40.

Hacein-Bey L, Harder DR, Meier HT, Lauer KK, Roman RJ: Improvement in angiographic vasospasm with a 20-HETE enzyme inhibitor in a subarachnoid hemorrhage dog model. Oral presentation, American Society of Neuroradiology 42nd Meeting, June 5-11, 2004, Seattle, WA, *Abstract #59, Proceedings of the American Society of Neuroradiology, p 46, 2004*

Hacein-Bey L, Roman RJ, Lemke DM, Lauer KK, Varelas PN, Torbey MT, Cusick JF, Harder DR: Evaluation of 20-hydroxyeiscosatetraenoic acid (20-HETE) levels in normal and abnormal blood and CSF. Oral presentation, American Society of

Neuroradiology 42nd Meeting, June 5-11, 2004, Seattle, WA, *Abstract #66, Proceedings of the American Society of Neuroradiology, p 51, 2004*

Jenkins A, Maxwell WL, and Graham DI (1989) Experimental intracerebral haematoma in the rat: sequential light microscopical changes. *Neuropathol Appl Neurobiol* **15:**477-86.

Kehl F, Cambji-Sapunar L, Maier KG, Miyata N, Kametani S, Okamoto H, Hudetz AG, Schulte ML, Zagorac D, Harder DR, and Roman RJ (2002) 20-HETE contributes to the acute fall in cerebral blood flow after subarachnoid hemorrhage in the rat. *Am J Physiol Heart Circ Physiol* **282:**H1556-H1565.

Kristian T, Gido G, Kuroda S, Schutz A, and Siesjo BK (1998) Calcium metabolism of focal and penumbral tissues in rats subjected to transient middle cerebral artery occlusion. *Exp Brain Res* **120:**503-509.

Lasker JM, Chen WB, Wolf I, Bloswick BP, Wilson PD, and Powell PK (2000) Formation of 20-hyroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney. Role of Cyp4F2 and Cyp4A11. *J Biol Chem* **275:**4118-4126.

Liu Y, Mituska S, Hashizume K, Hosaka T, and Nukui H (1996a) The time course of glucose metabolism in rat cerebral ischemia with middle cerebal artery occlusion-reperfusion model and the effect of MK-801. *Neurol Res* **18:**505-508.

Liu XH, Eun BL, Silverstein FS and Barks JD (1996b) The platelet activating factor antagonist BN 52021 attenuates hypoxic-ischemic brain injury in the immature rat. *Pediatric Res* 40:797-803.

Maier KG., Henderson L, Narayanan J, Alonso-Galicia M, Falck JR, and Roman RJ (2000) Fluorescent HPLC assay for 20-HETE and other P-450 metabolites of arachidonic acid. *Am J Physiol Heart Circ Physiol* **279:**H863-H871.

Miyata N, Taniguchi K, Seki T, Ishimoto T, Sato-Watanabe M, Yasuda Y, Doi M, Kametani S, Tomishima Y, Ueki T, Sato M, and Kameo K (2001) HET0016, a potent and selective inhibitor of 20-HETE synthesizing enzyme. *Br J Pharmacol* **133:**325-329.

Muthalif MM, Benter IF, Karzoun N, Fatima S, Harper J, Uddin MR, and Malik KU (1998) 20-HETE mediates calcium/calmodulin dependent protein kinase II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *Proc Natl Acad Sci USA* **95:**12701-12706.

Nagasawa H, and Kogure K (1989) Correlation between cerebral blood flow and histologic changes in a new rat model of middle cerebral artery occlusion. *Stroke* **20:**1037-1043.

Nakamura T, Sato M, Kakinuma H, Miyata N, Taniguchi K, Bando K, Koda A, and Kameo K. (2003) Pyrazole and isoxazole derivatives as new, potent, and selective 20-hydroxy-5,8,11,14- eicosatetraenoic acid synthase inhibitors. *J Med Chem* **46:**5416-5427.

Patel TR, Galbraith S, Graham DI, Hallak H, Doherty AM, and McCulloch J (1996) Endothelin receptor antagonist increases cerebral perfusion and reduces ischaemic damage in feline focal cerebral ischaemia. *J Cereb Blood Flow Metab* **16:**950-958.

Peeling J, Yan HJ, Chen SG., Campbell M, and Del Bigio MR (1998) Protective effects of free radical inhibitors in intracerebral hemorrhage in rat. *Brain Res* **795:**63-70.

Pilitsis JG, Coplin WM, O'Regan MH, Wellwood JM, Diaz FG, Fairfax MR, Michael DB, and Phillis JW (2003) Measurement of free fatty acids in cerebrospinal fluid from patients with hemorrhagic and ischemic stroke. *Brain Res* **985:**198-201

Randriambozvonjy V, Busse R and Fleming I (2003) 20-HETE-induced contraction of small coronary arteries depends on the activation of rho-kinase. *Hypertension* **41:**801-806.

Sarvary E, Halsey JH, Conger KA, Garcia JH, and Kovach AG. (1994) ATP and pH predictors of histologic damage following global cerebral ischemia in the rat. *Acta Physiol Hung* **82:**109-124.

Shino A, Matsuda M, Susumu T, and Handa J. (1991) Effects of the calcium antagonist nilvadipine on focal cerebral ischemia in spontaneously hypertensive rats. *Surg Neurol* **35:**105-110.

Shirakura S, Sano J, Karasawa A, and Kubo K. (1992) Protective effect of benidipine on arachidonic acid-induced acute cerebral ischemia in rats. *Jpn J Pharmacol* **59:**15-22.

Su P, Kaushal KM, and Kroetz DL (1998) Inhibition of renal arachidonic acid omega-hydroxylase activity with ABT reduces blood pressure in the SHR. *Am J Physiol Regulatory Integrative Comp Physiol* **275:**R426-38.

Sun CW, Falck JR, Harder DR, and Roman RJ (1999) Role of tyrosine kinase and PKC in the vasoconstrictor response to 20-HETE in renal arterioles. *Hypertension* **33:**414-418.

The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (1995) Tissue plasminogen activator for acute ischemic stroke. *N Eng J Med* **333:**1581-1587.

Tseng EE, Brock MV, Kwon CC, Annanata M, Lange MS, Troncoso JC, Johnston MV and Baumgartner WA (1999) Increased intracerebral excitatory amino acids and nitric oxide after hypothermic circulatory arrest. *Ann Thorac Surgery* **67:**372-376.

Wang MH, Brand-Schieber E, Zand BA, Nguyen X, Falck JR, Balu N, and Schwartzman ML (1998) Cytochrome P450-derived arachidonic acid metabolism in the rat kidney: characterization of selective inhibitors. *J Pharmacol Exp Ther* **284:**966-73.

Werling LL, Jacocks HM, Rosenthal RE and Fiskum G. (1993) Dopamine release from canine striatum following global cerebral ischemia/reperfusion. *Brain Res* **606:**99-105.

Yu M, Cambj-Sapunar L, Kehl F, Maier KG, Takeuchi K, Miyata N, Ishimoto T, Reddy LM, Falck JR, Gebremedhin D, Harder DR, Roman RJ (2004) Effects of a 20-HETE antagonist and agonists on cerebral vascular tone. *Eur J Pharmacol* **486:**297-306.

Xu F, Straub WO, Pa W, Su P, Maier KG, Yu M, Roman RJ, Ortiz de Montellano PR, and Kroetz DL (2002) Antihypertensive effect of mechanism-based inhibition of renal arachidonic acid ω-hydroxylase activity. *Am J Physiol Regul Integr Comp Physiol* **283:** R710-R720.

Footnotes

This work was supported in part by a grant HL-59996 from the National Institutes of Health.

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Figures legends

Fig. 1. Chemical structure of TS-011 (*N*-(3-Chloro-4-morpholin-4-yl)phenyl-*N'*-hydroxy-imidoformamide)

Fig. 2. Effects of TS-011 on the metabolism of AA by microsomes prepared from human kidneys (N=6, a) and spontaneously hypertensive rats (N=6, b). Results are expressed as percent of control. Each point represents a mean value $\pm$ SE. Effects of TS-011 on the metabolism of AA by CYP4F2, CYP4A11, CYP4F3A and CYP4F3B enzymes responsible for the formation of 20-HETE in humans are presented in panels c-e. Results are expressed as percent of control. Each point represents a mean value $\pm$ SE from 3 replicate incubations.

Fig. 3. a) Effect of TS-011 on regional cerebral blood flow (rCBF) after subarachnoid hemorrhage (SAH). Rats were pretreated 30 min before SAH with TS-011 (0.001-0.1 mg/kg, *i.v.* bolus) or vehicle. SAH was induced by injecting 0.3 ml of autologous arterial blood into the cisterna magna over 10 min period. b) Effect of TS-011 on the intracranial pressure (ICP) in rats after SAH. * indicates a significantly difference from the vehicle-treated groups

Fig. 4. Effect of TS-011 on rCBF in rats with preexisting SAH. SAH was induced by injecting 0.3 ml of blood in the cisterna magna at baseline (0 min). Vehicle or TS-011 (0.1 mg/kg, i.v.) was injected 30 min before and 30 min later after SAH induction. * indicates a significant difference vs. sham.

Fig. 5. Effect of TS-011 (0.1 mg/kg, i.v.) on the concentration of 20-HETE in CSF 2h after induction of SAH in rats. CSF (0.1 ml) was collected 2 hours after intracisternal injection of aCSF or blood. Control values were obtained from sham-operated rats that were not injected with blood in the cisterna magna. Numbers in figure indicate No of vessels studies. * indicates a significant differences vs. aCSF control. # indicates a significant differences vs SAH group.

Fig. 6 Effect of TS-011 on infarct volume in the rat transient middle cerebral artery occlusion (MCAO) model of ischemic stroke. a) 2,3,5-triphenyltetrazolium chloride (TTC) stained sections showing example of lesions from a vehicle treated control rat and a rat treated with TS-011. b) Infarct volumes 24 hours after MCAO in vehicle-treated control and TS-011 treated rats. TS-011 or vehicle were administered 60 min after occlusion of the MCA. * indicates a significant difference from the corresponding value in the vehicle-treated group. The number in parentheses indicates the number of animals studied per group.

Fig. 7. Effect of an injection of 20-HETE into the carotid arteries of rats on cerebral infarct size. Rats were treated with bolus injection of vehicle (a) or 12 mg/kg 20-HETE (b) into the carotid artery.

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1

Effect of TS-011 on CYP-1A2-, CYP-2C9-, CYP-2C19-, CYP-2D6- and CYP-3A4-Catalysed Substrate Oxidation

	CYP Enzyme for Drug Metabolism				
CYP Enzyme	1A2	2C9	2C19	2D6	3A4
N	1[a]	1[a]	1[a]	1[a]	1[a]
Mean IC_{50} (μM)	69.8	>100	>100	>100	>100

[a] N=1 with 6 times observation

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2

Receptor Binding and Enzyme Inhibition Profiles of TS-011 at the concentration of 1 μM All experiments were performed 2 times.

Type of Assays	TS-011 (1 μM) Inhibition (%)	Reference Compounds	IC_{50} (nM)	Tissue Type
Adenosine A1	< 10	CPA	0.88	Rat cerebral cortex
Adenosine A2A	< 10	NECA	4.5	Rat striatum
Adrenaline alpha 1 (non-selective)	< 10	Prazocin	0.42	Rat cerebral cortex
Adrenaline alpha 2 (non-selective)	< 10	Yohimbine	66	Rat cerebral cortex
Adrenaline beta 1	< 10	Atenolol	1320	Rat heart
Angiotensin (AT1)	< 10	Saralasin	1.6	Rat lung
Angiotensin (AT2)	< 10	Saralasin	2.8	Bovine cerebellum
ANP	< 10	ANP	0.11	Guinea pig cerebellum
Benzodiazepine (BDZ, central)	< 10	Diazepam	25	Rat cerebral cortex
Bradykinin (B2)	< 10	NPC567	6.1	Human recombinant (CHO cell)
Cholecystokinin (CCK1)	< 10	Devazepide	0.62	Human recombinant (NIH3T3 cell)
CRF1	< 10	CRF	6.3	Rat pituitary gland
Dopamine (D1)	< 10	SCH23390	0.92	Rat striatum
Dopamine (D2)	< 10	(+) butaclamol	3.9	Rat striatum
Endothelin A	< 10	Endothelin-1	0.42	a-10 cells
GABA (non-selective)	< 10	GABA	29	Rat cerebral cortex
NMDA	< 10	CGS19755	1,380	Rat cerebral cortex
PDGF	< 10	PDGF BB	0.58	Balb/c 3T3 cells
IL-1 beta	< 10	IL-1Beta	0.039	Balb/c 3T3 cells
TNF-alpha	< 10	TNF-alpha	0.19	U937 cells
Histamine (H1 central)	< 10	Pyrilamine	1.2	Guinea pig cerebellum

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Type of Assays	TS-011 (1 μM) Inhibition (%)	Reference Compounds	IC_{50} (nM)	Tissue Type
Insulin	< 10	Insulin	12	Rat liver
Muscarine (non-selective)	12	Atropine	0.65	Rat cerebral cortex
Neurokinin (NK1)	< 10	$[Sar^{9},Met(O_2)^{11}]$-SP	1.5	U-373MG cells
Neuropeptide Y (non-selective)	12	NPY	0.91	Rat cerebral cortex
Nicotine (Neuronal)	< 10	Nicotine	28	Rat cerebral cortex
Opiate (non-selective)	15	Naloxone	1.6	Rat cerebral cortex
PAF	< 10	WEB2086	3.9	Rabbit platelet
PCP	< 10	MK801	2.4	Rat cerebral cortex
5-HT (non-selective)	< 10	Serotonin	1.9	Rat cerebral cortex
5-HT1B	< 10	Serotonin	20	Rat cerebral cortex
5-HT2A	< 10	Ketanserin	1.7	Rat cerebral cortex
Sigma (non-selective)	< 10	Haloperidol	72	Rat cerebral cortex
Glucocorticoid	< 10	Dexamethasone	117	L-929 cells (cytosol)
VIP1	14	VIP	1.6	HT-29 cells
L-type Ca^{2+}-channel	< 10	Nitrendipine	16	A7r5 cells
COX1	< 10	Diclofenac	85	Rat seminal vesicle
COX2	11	NS398	5,200	Sheep placenta
NOS inducible	< 10	L-NMMA	466,000	RAW264-7 cells
NOS constitutive	11	L-NMMA	280	HUVEC (endothelial cells)
Phosphodiesterase I	< 10	8-methoxy-IBMX	1,800	Bovine brain
Phosphodiesterase III	< 10	Milrinone	220	Guinea pig heart
Phosphodiesterase IV	< 10	Rolipram	200	U-937 cells
Phosphodiesterase V	< 10	Dipyridamole	230	Human platelets
Aldose reductase	< 10	ICI-128,436	4.1	Bovine lens
ACE	< 10	Captopril	1.5	HUVEC
Caspase-3	< 10	DEVD-CHO	230	U937 cells

ACE = angiotensin-converting enzyme; ANP = atrial natriuretic peptide; CHO = Chinese hamster ovary; CPA = N6-cyclopentyladenosine; CRF = corticotropin-releasing factor; GABA = gamma-aminobutyric acid; IL = interleukin; NECA = 5N-ethylcarboxamide-adenosine; L-NMMA= (G)-monomethyl-

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

L-arginine; NMDA = N-methyl-D-aspartate; NPY = neuropeptide Y; PAF = platelet activating factor; PDGF = platelet-derived growth factor; TNF = tumor necrosis factor.

Receptor binding study; Adenosine A1 to L-type Ca^{2+}channel.

Enzyme inhibition study; COX-1 to caspase-3.

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3 Neurologic Deficit and Infarct Volume in 20-HETE-induced Stroke Model in Rats

Treatment	Neurologic Deficit (N=6)	Infarct Volume (N=6)
	24 hr	24hr
Vehicle	0 ± 0	0 ± 0
20-HETE		
8 mg/kg	10 ± 2	201.2 ±129.7
12 mg/kg	12 ± 1	215.2 ± 68.8

JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Table 4 Effect of a Bolus IV Injection of TS-011 on Cerebral Infarct Area and Neurologic Deficit 24 Hours after Intrastriatal Injection of Collagenase in Rats

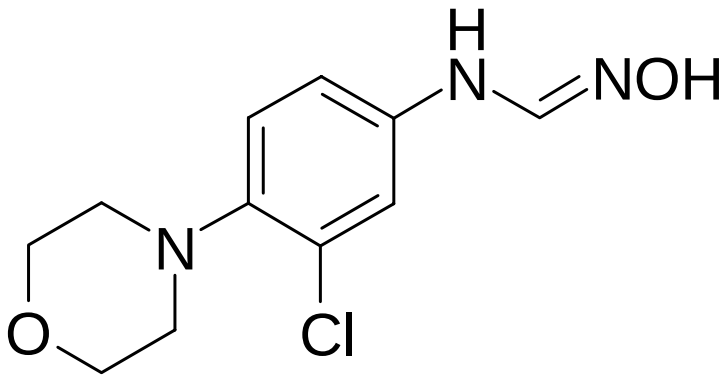
Drug (mg/kg)	Necrotic Area (mm^2) (N=10)	Neurologic Deficit (N=20)
Sham	0.00	0
Vehicle control	42.48 ± 5.14	7±0
TS-011 0.001	38.10 ± 3.68	7±0
0.01	30.10 ± 3.94	6±0
0.1	27.30 ± 3.83 *	5±0 #

* P < 0.05, significantly different from the vehicle control group (Dunnett's test)

# P < 0.01, significantly different from the vehicle control group (Steel test)

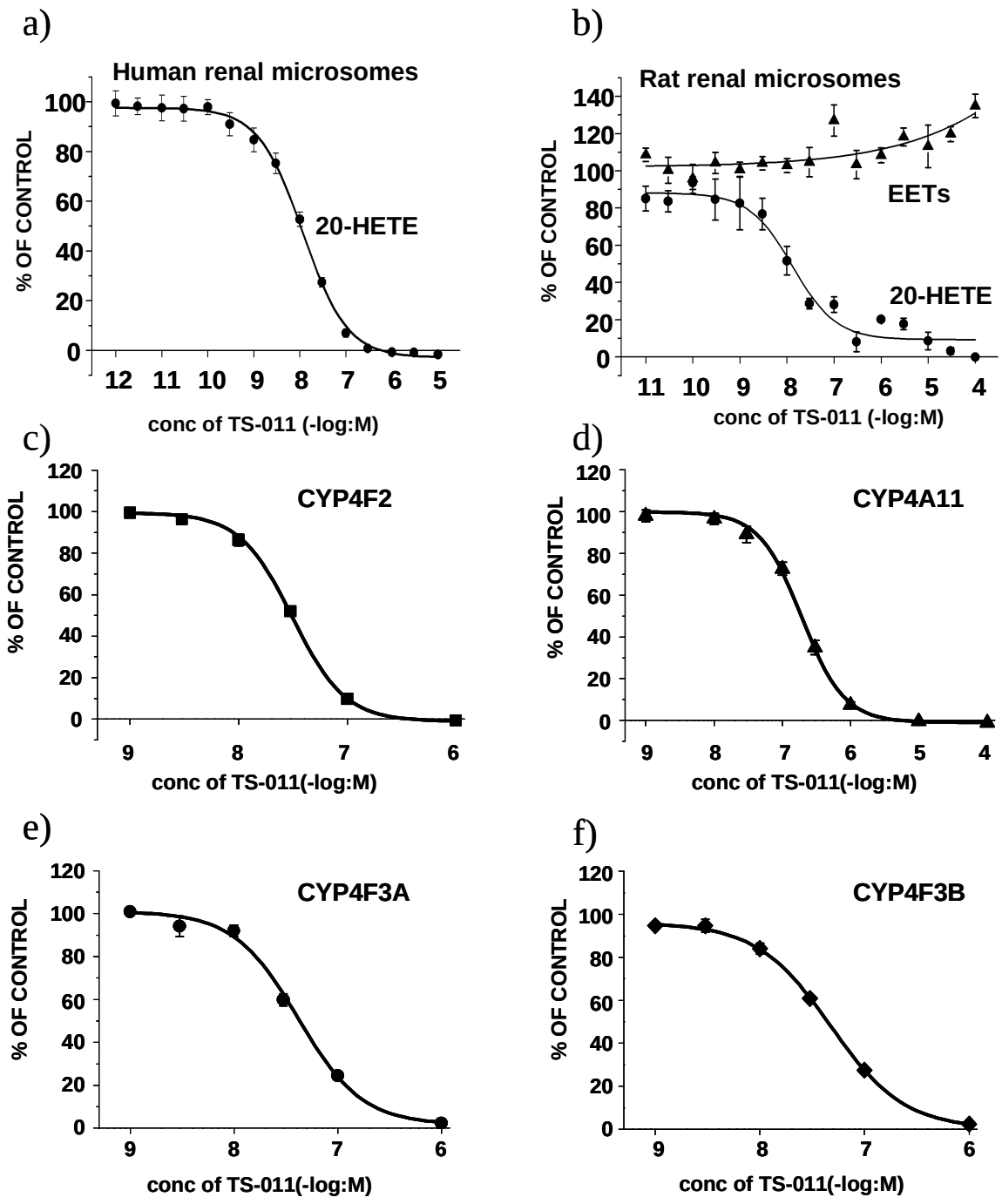
JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 1



JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 2

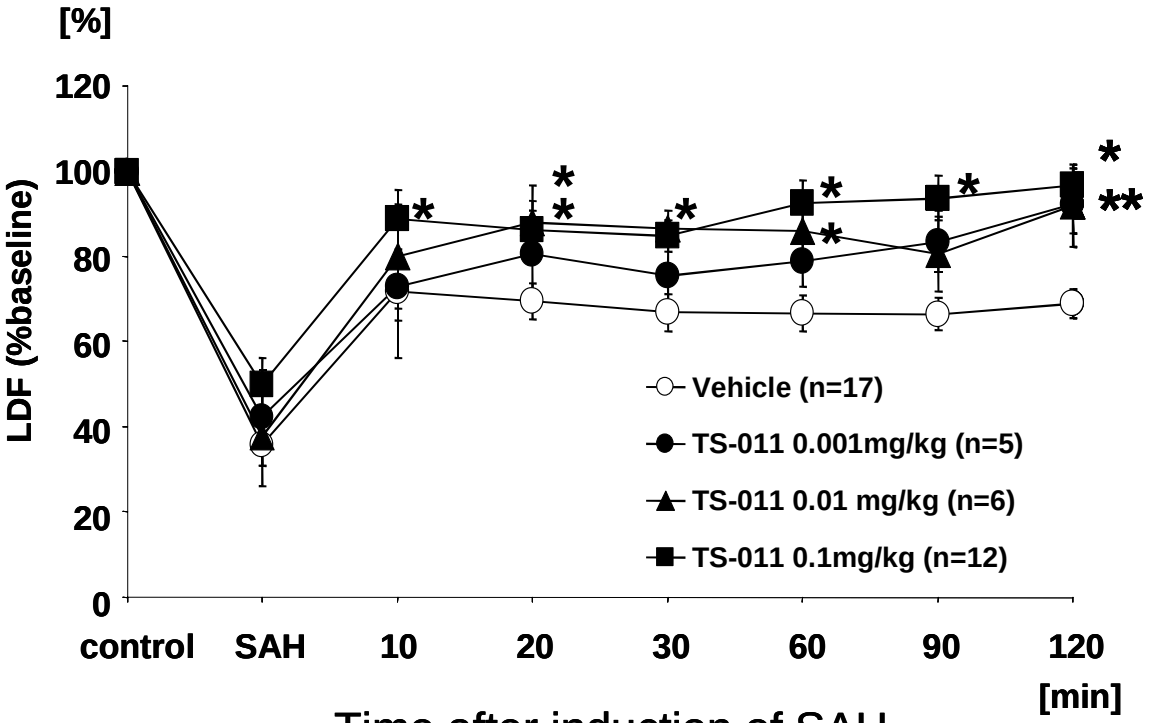


JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

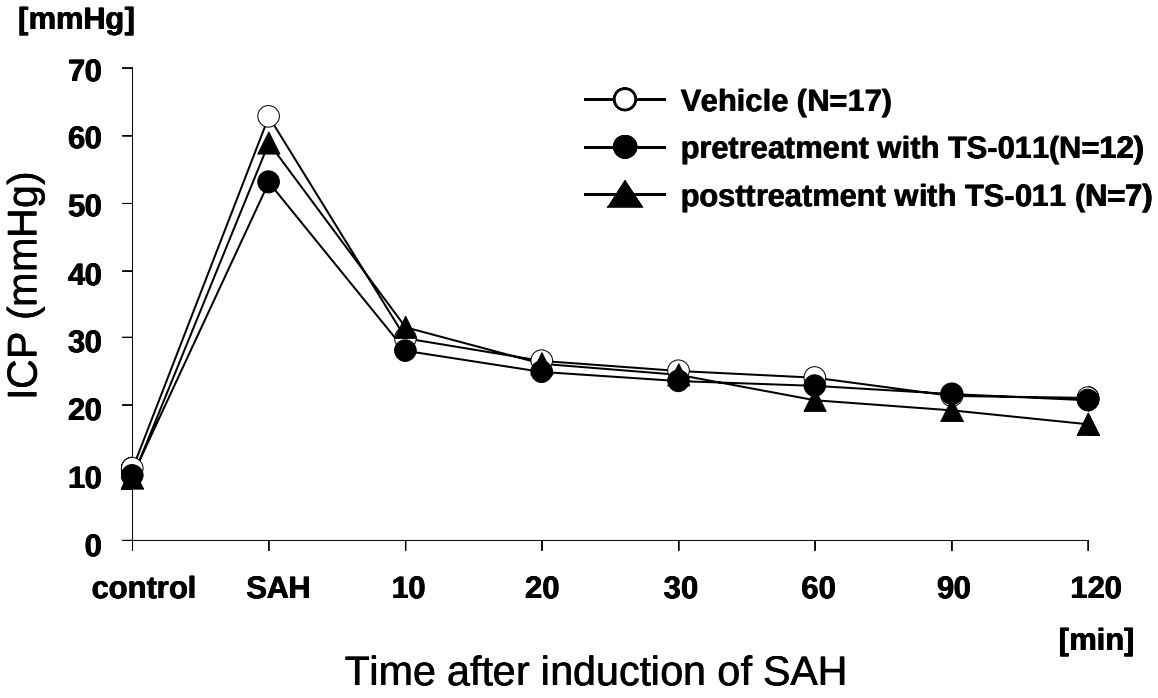
JPET#83964

Fig. 3

a)

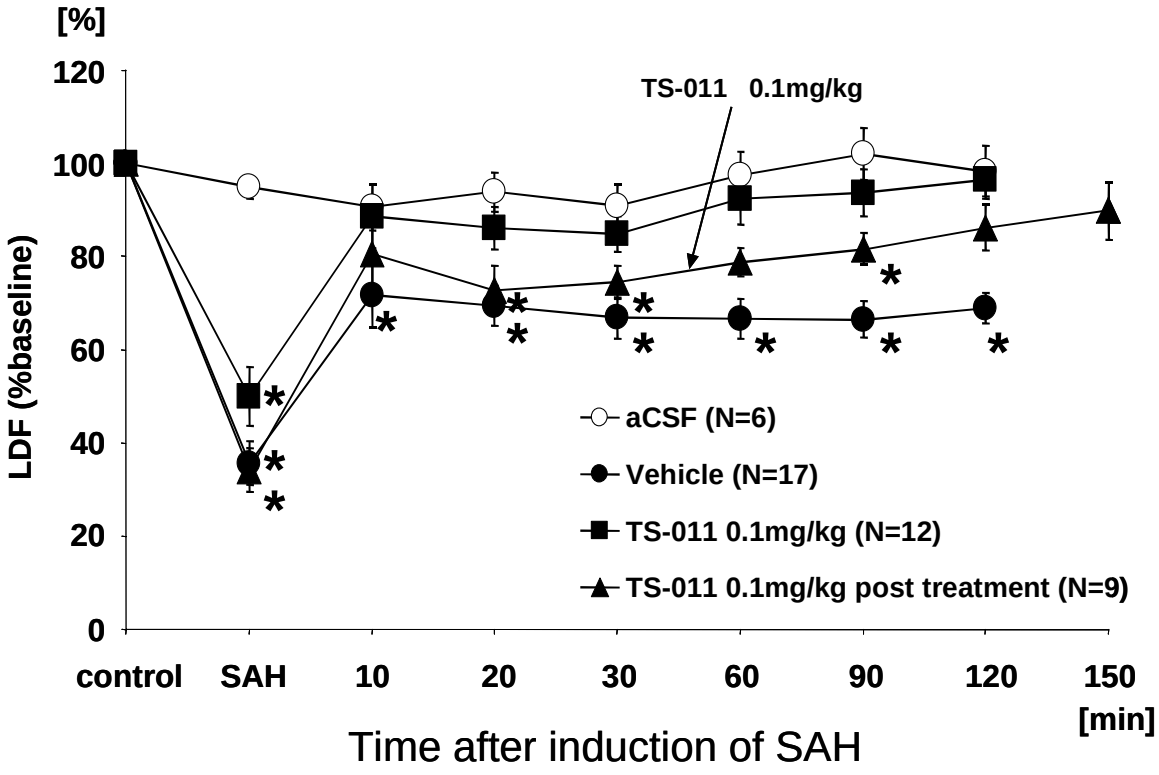


b)



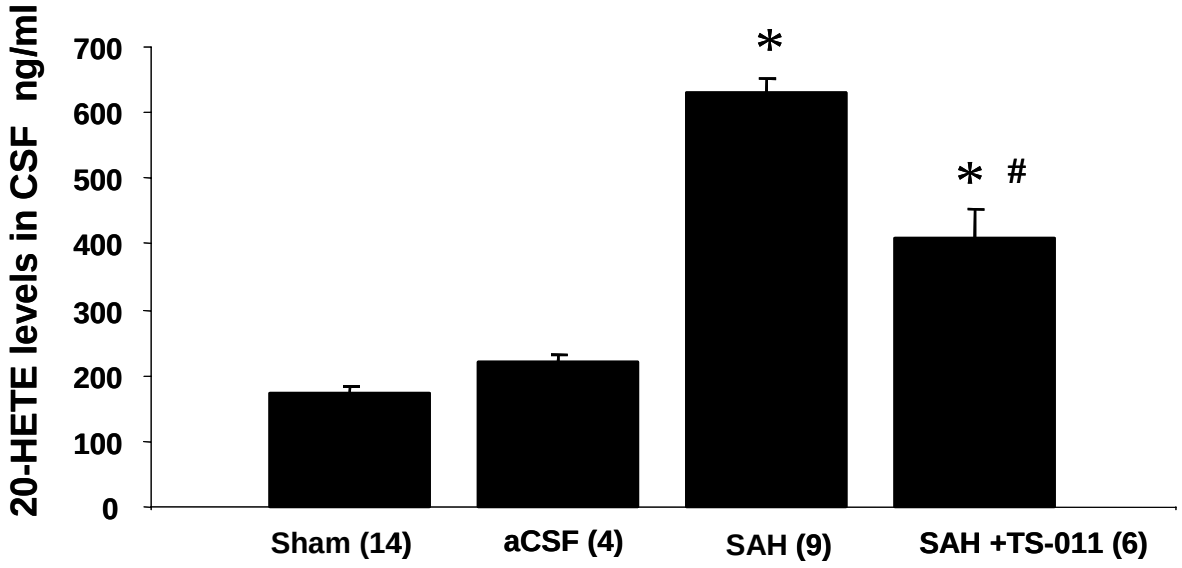
JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4



JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

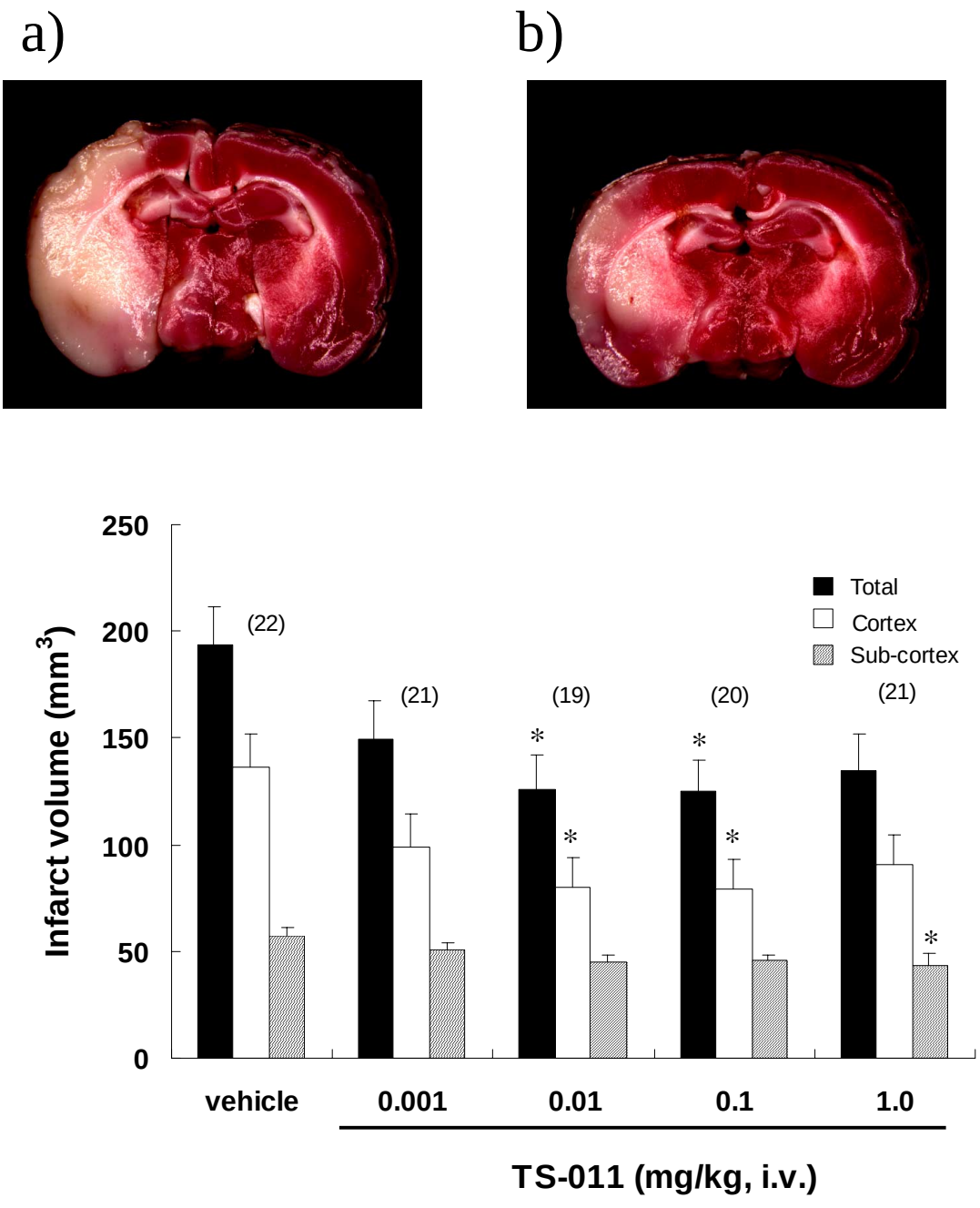
Fig. 5



JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

JPET#83964

Fig. 6



JPET Fast Forward. Published on April 14, 2005 as DOI: 10.1124/jpet.105.083964
This article has not been copyedited and formatted. The final version may differ from this version.

JPET#83964

Fig. 7

a) b)