

p38 Inhibitor Protects The Brain

Three distinct but interlinked mitogen-activated protein kinase (MAPK) pathways have been characterized. Many neurotrophins/growth factors bind to tyrosine kinase receptors and signal through Ras to the *extracellular signal-regulated kinase (ERK) MAPK* pathway, and this signaling can mediate neuronal development, growth, survival, and protection (Seger and Krebs, 1995; Skaper and Walsh, 1998). The *stress-activated MAPKs* [p38 and Jun N-terminal kinase

(JNK)] comprise the other two pathways. p38 and JNK play important roles in transducing stress-related signals by phosphorylating intracellular enzymes and transcription factors (Seger and Krebs, 1995; Robinson and Cobb, 1997) involved in cell survival, apoptosis, and inflammatory cytokine production (Lee et al., 1993; Xia et al., 1995; Lee and Young, 1996; Kummer et al., 1997).

Cerebral ischemic stroke is a powerful and destructive stimulus that produces significant changes in gene expression and enzyme activation that impact on the evolution of brain injury. Inflammatory mediators, brain inflammation, and apoptosis have all been shown repeatedly to contribute significantly to ischemic stroke injury and its outcome (Barone, 1998; Barone and Feuerstein, 1999). Currently available

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; CSAID, cytokine suppressive anti-inflammatory drug; SHR, spontaneously hypertensive rats; OGD, oxygen-glucose deprivation; MABP, mean arterial blood pressure; HR, heart rate; BT, body temperature; LDF, laser-doppler flow; TNF, tumor necrosis factor; LC/MS/MS, liquid chromatography/tandem mass spectrometry; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; BP, blood pressure.

therapies are only suitable in a small number (<2%) of patients (Fisher and Bogousslavsky, 1998; Atkinson and DeLemos, 2000), therefore new approaches to stroke intervention are clearly required. Since available data indicate that inflammatory mediator- and apoptosis-associated pathways provide novel targets to protect the brain in stroke, we were interested in investigating the inhibition of MAPK intracellular signaling pathways.

Although activation of the ERK MAPK pathway has been shown to be protective to brain cells (Murray et al., 1998; Anderson and Tolkovsky, 1999; Hetman et al., 1999; Singer et al., 1999), other data also demonstrate neuronal/brain protection by inhibition of the ERK path (Runden et al., 1998; Alessandrini et al., 1999). A balance between ERK and stress-activated MAPKs has been suggested to mediate cell survival (Xia et al., 1995; Heidenreich and Kummer, 1996). Also, sustained activation of JNK and p38 MAPK has been shown to be associated with neuronal death/apoptosis (Yang et al., 1997; Horstmann et al., 1998; Maroney et al., 1998; Skaper and Walsh, 1998; Harada and Sugimoto, 1999; Le-Niculescu et al., 1999), and selective p38 MAPK inhibitors can promote the survival of a variety of neurons in vitro (Xia et al., 1995; Kummer et al., 1997; Horstmann et al., 1998; Skaper and Walsh, 1998; Harada and Sugimoto, 1999). p38 MAPK activation has been shown to be involved in glutamate toxicity-induced neuronal apoptosis (Kawasaki et al., 1997). In global forebrain ischemia, p38 MAPK activation has been identified in microglial cells adjacent to dying, vulnerable neurons (Walton et al., 1998). Both the ERK and p38 MAPK pathways have been shown to be activated in vivo poststroke (Alessandrini et al., 1999; Irving et al., 2000).

One class of p38 inhibitor compounds, the pyridinyl imidazoles (originally named CSAIDs for "cytokine-suppressive anti-inflammatory drugs"), have well characterized therapeutic utility related to their inhibition of TNF α and interleukin-1 β production (Lee et al., 1994; Boehm et al., 1996; Lee and Young, 1996; Kumar et al., 1997; Young et al., 1997). This can reduce inflammation, including the expression of other inflammatory mediators/proteins, thus significantly affecting the ultimate degree of tissue injury. CSAIDs inhibit the catalytic activity of activated/phosphorylated p38 to phosphorylate MAPKAP-K2, which upon activation serves in nuclear import/export of p38 (and itself) and provides for the phosphorylation of downstream substrates (e.g., Hsp27 for MAPKAP-K2) in the cytoplasm (Ben-Levy et al., 1998). Not only does p38 phosphorylation/activation phosphorylate transcription factors (e.g., ATF2), it can also up-regulate protein transcription and translation and stabilize mRNA (Lee and Young, 1996).

Recently, we (Irving et al., 2000) have shown significant activation of p38 in ischemic brain areas exhibiting evolving brain injury in the rat. Intense activation was observed over the initial 6 h following stroke, and activation extended for as long as 24 h poststroke in areas adjacent to brain infarction. Here, we characterize the activity of SB 239063 (Fig. 1), a second-generation p38 MAPK inhibitor (Adams et al., 1998), in vivo and in vitro. SB 239063 exhibits potent inhibition of p38 activity and has improved selectivity and cellular and in vivo activity over previous p38 inhibitors (e.g., the widely studied first-generation inhibitor, SB 203580; Badger et al., 1996). Also, we demonstrate that oral and intravenous dosing regimens of SB 239063 that relate to its anticytokine and

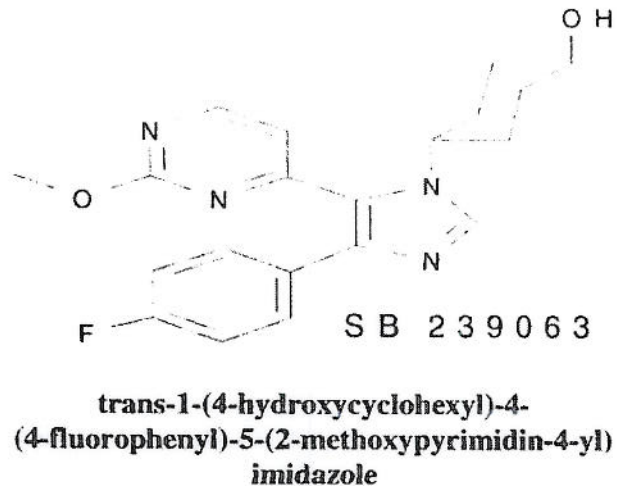


Fig. 1. Chemical structure of the second-generation CSAID, SB 239063 [*trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyrimidin-4-yl)imidazole].

anti-inflammatory activities and that are based on pharmacokinetics and established cellular potency can protect the brain from injury and improve neurologic functional outcome following stroke.

Materials and Methods

Animal Research Guidelines. All procedures on animals and their housing and care were in accordance with the *Guide For The Care and Use of Laboratory Animals* (Bethesda, MD: Office of Science and Health Reports, Division of Research Resources/National Institutes of Health; 1985. U.S. Department of Health, Education, and Welfare (Department of Health and Human Services) publication 85-23) and the UK Animals (Scientific Procedures) Act (1986). Procedures using laboratory animals were approved by the U.S. and UK internal Institutional Animal Care and Use Ethics Committee of SmithKline Beecham Pharmaceuticals.

Enzyme and Cell-Based Assays. Both SB 203580 and SB 239063 were evaluated for their inhibitory activity and selectivity on a series of isolated MAPKs. p38 (four isoforms: α , β , γ , and δ), MEK, ERK, MAPKAP-K2, JNK-1, and c-Raf were cloned, expressed, and purified at SmithKline Beecham Pharmaceuticals and assayed under optimum conditions for IC₅₀ (μ M) determinations (Lee et al., 1994; Young et al., 1997). In addition, the inhibitory (IC₅₀ in μ M) effects of both compounds on lipopolysaccharide (LPS)-stimulated human monocyte TNF α production was determined in vitro as described previously (Cuenda et al., 1995; Lee and Young, 1996).

In Vivo Pharmacodynamic and Inflammation Assays. The inhibitory activity (IC₅₀ in mg/kg, p.o.) on plasma TNF α production in Lewis rats injected with LPS was determined by enzyme-linked immunosorbent assay as described previously (Badger et al., 1996). Doses of SB 203580 and SB 239063 were administered orally in acidified 0.5% tragacanth (as 10 ml/kg) 30 min before the injection of LPS (30 μ g/kg, i.p.). Vehicle (acidified 0.5% tragacanth) was administered as control. Also, the inhibitory activity on adjuvant arthritis was determined as described previously (Badger et al., 1996). Adjuvant arthritis was produced by a single injection of 0.75 mg of *Mycobacterium butyricum* (Difco, Detroit, MI) suspended in paraffin oil into the base of the tail of male Lewis rats, 6 to 8 weeks old (160–180 g) on day 0. Hindpaw volumes were measured by water displacement on day 22. Doses of SB 203580, SB 239063, or vehicle were administered orally 30 min before *M. butyricum* on day 0 and

sus the control/vehicle treatment.

SB 239063 Pharmacokinetics Used to Determine Intravenous Dosing Regimen(s). The pharmacokinetic profile of SB 239063 was determined in male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 300 to 350 g. SB 239063 (0.9 mg/kg) was administered as a 30-min i.v. infusion (4 ml/kg total volume), and blood samples were obtained from a lateral tail vein at various time points up to 8 h after dosing. Fifty-microliter aliquots of plasma were isolated by centrifugation and frozen until analysis. For initial quantitative analysis, analyte was isolated from rat plasma by acetonitrile precipitation. Concentrations of SB 239063 were determined in each sample by LC/MS/MS with TurboIonSpray interface; using 50 μ l of plasma, the lower limit of quantitation of the assay was 10.0 ng/ml. Standard noncompartmental techniques were used for pharmacokinetic data (Gibaldi and Perrier, 1986). These pharmacokinetic parameters then were used to design steady-state infusion regimens that would result in target plasma concentrations of 1 to 4 μ M (i.e., in the range of 0.38–1.5 μ g/ml) by calculating drug infusion rate by multiplying desired steady-state concentration by the measured plasma clearance. Target plasma concentrations were selected from the cell-based data indicating that these concentrations should provide robust inhibition of p38's actions (i.e., plasma levels should be in the 1–4 μ M range; see below and *Results*).

Using these calculated values based on the pharmacokinetic data, intravenous infusions of SB 239063 solutions were administered for 6 h to provide inhibition during the period of initial, intense p38 activation following stroke (Irving et al., 2000). Infusions of drug were provided to target plasma concentrations of 0.38, 0.75, and 1.5 μ g/ml as listed in Table 1. Rats were anesthetized with pentobarbital (65 mg/kg, i.p.), and SB 239063 was dissolved in acidified isotonic saline and delivered via calibrated infusion pumps (Harvard Apparatus Inc., Holliston, MA) into the femoral or tail vein. In separate groups of rats, blood samples and total forebrains were removed from rats after 1 and 6 h of SB 239063 infusion and 24 h after initiation of the 6-h SB 239063 infusions as described in Table 1. In addition, in other groups of rats, blood samples and ischemic and control forebrain hemispheres were collected after 6-h SB 239063 infusions that began 15 min poststroke (i.e., as carried out in the neuroprotection studies described below). SB 239063 brain and plasma concentrations were measured using LC/MS/MS as described above.

Focal Ischemia. Focal cerebral ischemia was produced in two stroke models (i.e., referred to here as moderate and more severe brain injury models independently conducted in two different laboratories; these models are routinely used to evaluate the robustness of stroke targets/drug treatments within SmithKline Beecham Pharmaceuticals). For moderate brain injury, focal ischemia experiments were performed on male spontaneously hypertensive rats (SHR; Taconic Farms, Germantown, NY) weighing 290 to 340 g. SHR were chosen because they exhibit a moderate but more consistent degree of brain damage (i.e., restricted to the cortex) following permanent or

electrocoagulation of the MCA. In addition, following permanent MCAO, they do not exhibit morbidity/mortality (Barone et al., 1992). Body temperature was maintained at 37°C during all surgical procedures and during recovery from anesthesia (i.e., until normal locomotor activity returned). Animals were anesthetized with pentobarbital (65 mg/kg, i.p.) and they underwent permanent, right middle cerebral artery occlusion (MCAO) for 24 h as described previously (Barone et al., 1992, 1998). Briefly, rats were positioned in a stereotaxic unit and following a right craniotomy and removal of the dura mater, the bent tip of a platinum-iridium wire was placed under the middle cerebral artery at the level of the inferior cerebral vein using a micromanipulator, and the artery was occluded and cut at this distal location using electrocautery.

An initial study involved oral dosing of SB 239063 as a stroke protection follow-up to the earlier oral pretreatment work in inflammation models (i.e., before intravenous dose regimen/formulation studies that were based on the pharmacokinetic data using the drug for postischemia intervention treatment as described below). In this initial study, oral SB 239063 was administered (i.e., vehicle/acidified 0.5% tragacanth, 5, 15, 30, or 60 mg/kg as 10 ml/kg) at 1 h pre- and 6 h following the production of moderate stroke in SHR as described above. In intravenous studies, SB 239063 was administered intravenously for 6 h post-MCAO starting 15 min following the onset of ischemia to provide targeted plasma levels of 0.38, 0.75, and 1.5 μ g/ml (as described above and in Table 1). Control animals for this study received i.v. administration of vehicle (acidified saline) for 6 h at the same flow rate used for SB 239063. The 6-h infusion period was used to cover the period of initial, intense activation of p38 that was identified poststroke in our previous work (Irving et al., 2000). All drug dosages/regimens were assigned to animals in a counterbalanced manner to control for drug treatment-time of day effects. Twenty-four hours after onset of focal ischemia, each rat was then evaluated for neurological deficits using two graded scoring systems as previously described (Barone et al., 1992, 1998). Briefly, forelimb scores were 0 (no observable deficit), 1 (any contralateral forelimb flexion when suspended by the tail), and 2 (reduced resistance to lateral push toward the paretic, contralateral side). A hindlimb placement test consisted of pulling the contralateral hindlimb away from the rat over the edge of a table. A normal response (0 score) is an immediate repositioning of the limb back onto the table, and an abnormal/deficit response (1 score) is no limb placement/movement. The total score (i.e., the sum) of both tests was used as a global neurological deficit grade for each rat. Rats were then (i.e., 24 h poststroke) euthanized by an overdose of sodium pentobarbital (200 mg/kg, i.p.). The brains were immediately removed, and 2-mm coronal sections were cut from the entire forebrain area (i.e., from the olfactory bulbs to the cortical-cerebellar junction) using a brain slicer (Zivic-Miller Laboratories, Portersville, PA). The coronal sections were immediately stained in a solution of 1% triphenyltetrazolium chloride, transferred to 10% formalin (in 0.1% sodium phosphate

TABLE 1
In vitro and in vivo activities of SB 239063 compared with SB 203580 (i.e., a second- vs. first-generation p38 inhibitor)

Assay	SB 203580	SB 239063
Inhibition of isolated enzyme activity (IC ₅₀ in μ M)		
p38 α	0.040 ^b	0.044
MEK	>10	>10
ERK	>10	>10
MAPKAP-K2	>10	>10
JNK-1	5	>10
c-Ref	0.4	>10
In vitro inhibition of LPS-induced TNF α production in human monocytes (IC ₅₀ in μ M)	1.00	0.35
In vivo inhibition of LPS-induced TNF α production in rat plasma (IC ₅₀ in mg/kg, p.o.)	25.0	2.6
In vivo inhibition of adjuvant-induced arthritis in the rat (percent inhibition at mg/kg, p.o. dose treatment)	60% at 60 45% at 30 0% at 10	60% at 30 51% at 10 28% at 3

^a Values listed are for p38 α . Similar results were obtained for p38 β . Neither compound had any inhibitory activity (i.e., no effects at 10 μ M on p38 γ or p38 δ).

^b For determinations of IC₅₀ or percentage inhibition, the number of preparations or animals used was four to nine.

DAHER) for at least 20 min. Briefly, brain injury was quantified using an *Optimas image analysis system* (DataCell, Berkshire, UK), and the degree of brain damage was corrected for the contribution made by brain edema/swelling as described previously (Barone et al., 1998). Hemispheric swelling, infarct volume (mm^3) was calculated from the infarct areas measured from the sequential forebrain sections, and infarct size was expressed as the percentage of infarcted tissue in reference to the contralateral hemisphere.

For a more severe focal ischemia that produced a greater extent of brain injury, focal stroke was produced in male Sprague-Dawley rats (Charles River, Kent, UK), weighing 300 to 350 g. Rats were anesthetized with halothane (4% for induction and 1.0–1.5% for maintaining constant depth) in nitrous oxide/oxygen (70:30%) and body temperature was maintained at 37°C using a thermometer-controlled heating blanket. The left MCA was occluded using the intraluminal suture technique described previously (Zea Longa et al., 1980; Rogers et al., 1997). Briefly, the common carotid, external carotid, and internal carotid arteries were exposed through a midline cervical incision. The tip of a 30-mm length of 3-0 monofilament nylon suture was heat-blunted to a diameter of 0.28 to 0.30 mm and coated with poly(L-lysine) (Bayalev et al., 1995). The suture then was advanced 18 to 20 mm from the external carotid artery until mild resistance was met (i.e., indicating occlusion of the origin of the MCA). The nylon suture was secured in this position, and it remained in place until animals were euthanized. After surgery, the rats were allowed to recover in an incubator and were then housed overnight in individual cages. SB 239063 was administered as described for moderate focal ischemia (above). Neurological evaluations, carried out immediately before euthanasia 24 h poststroke, were modified from a behavioral rating scale as described previously (Mackay et al., 1996): 0 = no neurological deficit; 1 = failure to extend right forepaw fully; 2 = decreased grip of right forepaw while tail is gently pulled; 3 = contralateral circling or walking; 4 = walks only when stimulated; 5 = unresponsive to stimulation with a depressed level of consciousness. Twenty-four hours after MCAO, rats were deeply anesthetized with halothane and transcardially perfused with 4% neutral buffered formalin containing 5% sucrose. The brains were postfixed for 48 h and then removed from the skull and processed for histological quantification of ischemic damage similar to that described previously (Mackay et al., 1996; Rogers et al., 1997) and similar to that described above for moderate stroke. Briefly, the forebrain was cut serially at 1.5-mm intervals into 50- μm coronal sections and stained with 1% cresyl fast violet (Sigma Chemical Co., St. Louis, MO). Sections that corresponded most closely to eight stereotaxically predetermined forebrain coronal planes were examined. Areas of the brain with reduced staining (i.e., infarcted brain) were quantified using an *Optimas image analysis system* (DataCell), and infarct volume was calculated from the infarct areas on sequential forebrain sections (mm^3); the degree of brain damage/infarction was corrected for the contribution of edema/swelling as described previously. Percentage of hemispheric swelling and infarction in reference to the contralateral hemisphere was also determined.

Cortical Perfusion, Hemodynamics, and Body Temperature. Male SHR rats (333 ± 5 g) were anesthetized with sodium pentobarbital (65 mg/kg, i.p.), and the femoral artery was cannulated with polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) and attached to a DTX Plus transducer (Ohmeda, Singapore) for monitoring of arterial blood pressure. Cortical perfusion was measured as described previously (Barone et al., 1992, 1998). Briefly, rats were placed on a thermal heating pad and positioned in a stereotaxic unit where a 2- to 3-mm diameter craniotomy was made in the skull (centered at anteroposterior = 0 mm, lateral = 4 mm from bregma with level skull). The probe (1 mm in diameter) of a laser-doppler perfusion monitor (Periflux PF3, Perimed, Inc., Stockholm, Sweden) then was positioned on the surface of the dura. The probe was adjusted using a micromanipulator to give optimum local cortical

monitor body temperature. Laser-doppler flow (LDF) and arterial blood pressure (BP) were recorded continuously on an Astro-Med (West Warwick, RI) model 7400 physiological recorder. Heart rate (HR) was counted using the arterial wave form. Each animal was allowed to stabilize for 15 min to assure consistent LDF and BP readings before either vehicle (acidified saline, $n = 6$) or SB 239063 ($n = 5$) was infused via the tail vein (1.5 ml/h, 1.0 mg/ml). Measurements of cortical perfusion, mean arterial blood pressure (MABP), HR, and body temperature (BT) were taken at baseline (start of infusion), 20, 40, and 60 min (end of infusion).

Organotypic Hippocampal Slice Culture. Organotypic hippocampal cultures are an intermediate between the *in vivo* models and primary neuronal cultures but provide a method of determining direct neuronal effects of a drug on ischemic injury. The majority of glial-neuronal interactions and cell stoichiometry are maintained, making investigations of these mechanisms of cell death similar to *in vivo* but in absence of the circulation and infiltrating cells. Slice cultures were prepared from 8-day-old Sprague-Dawley rat pups as described previously (Vornov et al., 1994). Pups were killed by decapitation, and the hippocampi were dissected out. Using a McIlwain tissue chopper (TPI/Vibratome, St. Louis, MO) 400- μm thick slices were cut and then placed into ice-cold growth medium, and after 9 to 12 days the cultures were viable for use in oxygen-glucose deprivation (OGD) (i.e., *in vitro* ischemia) experiments. Cultures were placed in serum-free medium 1 h pre-OGD. The cultures were then transferred to six-well plates containing glucose-free medium saturated with 95% N_2 , 5% CO_2 and placed into an anaerobic chamber that was equilibrated to 37°C, 100% humidity, 95% N_2 , 5% CO_2 was blown through the chamber for 10 min before the chamber was sealed for a 45-min period of OGD. Several concentrations of SB 239063 (0–50 μM ; each in duplicate; $n = 9$ separate experiments) were present for 1 h before and during the 45 min of OGD. On removal of the plates from the chamber (i.e., at the end of OGD), the inserts were transferred to prewarmed serum-free medium containing 6 $\mu\text{g/ml}$ propidium iodide and then placed back into the CO_2 incubator. After 23 h, analysis of damaged CA1 hippocampal neurons was carried out using NIH IMAGE 1.62. Data was normalized to percentage of total CA1 neurons damaged in each culture.

Statistical Analysis. Results are presented as mean \pm S.E.M. and/or median \pm 25:75% median range. IC_{50} determinations were made by linear interpolation. Statistical analyses of parametric data were carried out using ANOVA with least significant difference follow-up testing or *t* test, if appropriate. Statistical analyses of nonparametric data were carried out using the Kruskal-Wallis (ANOVA) test with Mann-Whitney *U* test(s) follow-up testing (controlling type I error at $p < 0.05$) or simply a Mann-Whitney *U* test (i.e., if appropriate). Differences between groups (as outlined under *Results*) were considered significant if $p < 0.05$.

Results

p38 Enzyme Activity, Kinase Selectivity, and Cell-Based Cytokine Inhibition. We characterized the *in vitro* and *in vivo* activity profile of the more recently discovered CSAID, SB 239063. SB 239063 produced an ATP competitive inhibition of isolated p38 MAPK with an IC_{50} of 44 nM (Table 1). It also exhibited increased selectivity against a panel of protein kinases that exceeds previous p38 inhibitors (e.g., SB 239063 was compared with SB 203580, which is a prototype first-generation CSAID) and displayed increased (i.e., 3-fold) potency at inhibiting TNF α production in LPS-stimulated monocytes *in vitro*, thus exhibiting an improved enzyme and cellular activity profile (Table 1).

In Vivo Pharmacodynamic and Inflammation Assays. SB 239063 also exhibited increased activity over SB

levels and in reducing adjuvant arthritis paw inflammation in the Lewis rat (Table 1). Generally, SB 239063 exhibited a 3- to 10-fold increased *in vivo* potency over SB 203580.

Oral SB 239063 Neuroprotection from Focal Ischemic Brain Injury. The improved activity and remarkable *in vivo* activity of SB 239063 in LPS-induced TNF release and experimental inflammatory arthritis models prompted our evaluation of its oral administration in moderate focal stroke. Significant protection from brain injury and neurological deficits was in the same *in vivo* oral dose activity range (Table 2). At oral doses of 5, 15, 30, and 60 mg/kg, SB 239063 reduced infarct volume by 42% ($p < 0.01$), 48% ($p < 0.01$), 29% ($p < 0.05$), and 14%, respectively, and reduced neurological deficits by 31% ($p < 0.05$), 42% ($p < 0.01$), 23% ($p < 0.05$), and 12%, respectively. Effects on percentage of hemispheric infarction (infarct size normalized to the size of the normal contralateral hemisphere) were similar to those on infarct volume with vehicle, 5, 15, 30, and 60 mg/kg exhibiting percentage of hemispheric infarcts (and differences from vehicle) of 17.0 ± 0.9 , 10.6 ± 1.5 ($p < 0.01$), 9.2 ± 1.4 ($p < 0.01$), 12.5 ± 1.0 ($p < 0.05$), and 14.0 ± 0.9 .

Pharmacokinetics and Establishing SB 239063 Intravenous Dosing Regimen. Although the oral activity dose range was already available from the *in vivo* data in Tables 1 and 2, we set out to establish an efficacious intravenous dosing regimen based on pharmacokinetic data and known *in vitro* and *in vivo* activities. Therefore, we determined the SB 239063 intravenous pharmacokinetic profile to provide the information necessary to determine the administration regimens that would provide plasma drug concentrations at levels that would block the consequences of initial stroke-induced p38 activation. In this manner, we could use the improved p38 inhibitor to understand the role of p38 in brain injury. In general, *i.v.* administration of a neuroprotective drug is considered optimum for stroke intervention (e.g., it can provide for the rapid achievement and control of neuroprotective plasma levels poststroke). SB 239063 exhibited moderate clearance in the rat (33.7 ± 9.8 ml/min/kg) with a half-life of 74.4 ± 31.5 min and a large volume of distribution (3.0 ± 0.6 l/kg) (Fig. 2, top). Based on these pharmacokinetic parameters, infusion rates were estimated that successfully achieved target plasma levels of 0.38, 0.75, and 1.5 μ g/ml SB 239063 at the end of a 6-h infusion (Table 3). These levels (i.e., calculated to be from 1–4 μ M in the plasma) were expected to inhibit cellular p38 activity *in vivo* (e.g., based on the measured *in vitro* cellular inhibitory activity of SB 239063 as listed in Table 1). The SB 239063 6-h intravenous dose regimens produced linear (i.e., dose-proportional) changes and expected drug plasma concentrations that dissipated by 24 h. The 6-h plasma levels were not affected by

was approximately 4 to 5% of plasma levels and closely followed changes in plasma levels. Following 6-h MCAO, the ischemic hemisphere exhibited 31 to 36% increased drug concentration above that of the control hemisphere (Fig. 2, bottom).

Intravenous SB 239063 Neuroprotection from Focal Ischemic Brain Injury. Significant protection from brain injury and neurological deficits was demonstrated in both moderate and severe stroke models due to intravenous treatment with the selective p38 inhibitor SB 239063. In moderate stroke, targeted plasma concentrations of 0.38, 0.75, and 1.5 μ g/ml reduced infarct size by 23.3% ($p < 0.05$), 40.7% ($p < 0.01$), and 32.7% ($p < 0.05$) and reduced neurological deficits by 30.8% ($p < 0.05$), 34.6% ($p < 0.01$), and 11.5%, respectively (Fig. 3). Percentage of hemispheric infarct results for SB 239063 were identical to those of infarct volume (in mm³), with vehicle and 0.38, 0.75, and 1.5 μ g/ml SB 239063-treated rats exhibiting percentage of hemispheric infarcts (and differences from vehicle) of 18.5 ± 1.4 , 14.1 ± 1.1 ($p < 0.05$), 11.0 ± 2.0 ($p < 0.01$), and 12.7 ± 1.7 ($p < 0.05$), respectively. Neurological deficit results expressed as means or medians and analyzed by parametric or nonparametric ANOVA demonstrated SB 239063 neuroprotection. Hemispheric swelling (only a few percent in this moderate stroke model) was not affected by drug treatment (data not shown). In severe focal stroke (Fig. 4), targeted plasma concentrations of 0.38, 0.75, and 1.5 μ g/ml reduced the much larger infarcts by 20.9% ($p < 0.05$), 27.5% ($p < 0.05$), and 16.4% and reduced neurological deficits by 16.7%, 33.3% ($p < 0.05$), and 0%, respectively. Percentage of hemispheric infarct data for SB 239063 also was similar to that for infarct volume, with vehicle and 0.38, 0.75, and 1.5 μ g/ml SB 239063-treated rats exhibiting percentage of hemispheric infarcts of 47.7 ± 2.6 , 37.5 ± 3.1 , 35.1 ± 4.1 ($p < 0.05$), and 41.1 ± 2.3 , respectively. Most of the total brain injury protection from SB 239063 was due to reduced cortical injury. This is typical for neuroprotection in this severe focal stroke model. Hemispheric swelling was also reduced by drug treatment (data not shown), however, infarct size adjusted for swelling (i.e., when the effects of swelling were eliminated) was significantly reduced as described above.

SB 239063 Effects on Cortical Perfusion, Hemodynamics, and Body Temperature. Table 4 lists the absolute and percentages of change in cortical perfusion (LDF), blood pressure (MABP), HR, and BT observed during the infusion of SB 239063. Absolutely no effects were observed with infusion of SB 239063 using the same amount of drug delivered over 1 h as that delivered over 6 h in the middle (i.e., 0.75 mg/ml blood level) dose regimen that provided the greatest degree of neuroprotection in both stroke models.

TABLE 2
Oral SB 239063 produces neuroprotection in moderate stroke (SER MCAO)*

	Oral Dose (h.i.d.)				
	Vehicle	5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg
Infarct volume (mm ³)	148 ± 8	86 ± 12**	77 ± 11**	105 ± 8*	127 ± 8
Hemispheric infarct (%)	17.0 ± 0.9	10.6 ± 1.5**	9.2 ± 1.4**	12.5 ± 1.0*	14.0 ± 0.9
Neurological deficit [mean (median;75;25)]	2.6 ± 0.1 (3;2;3)	1.8 ± 0.3 (2;1.25;2)*	1.5 ± 0.2 (1.5;1.2)**	2.0 ± 0.2 (2;2.2)*	2.3 ± 0.2 (2;2;3)

* Number of rats in each group was 6 to 30.

* $p < 0.05$ different from vehicle.

** $p < 0.01$ different from vehicle.

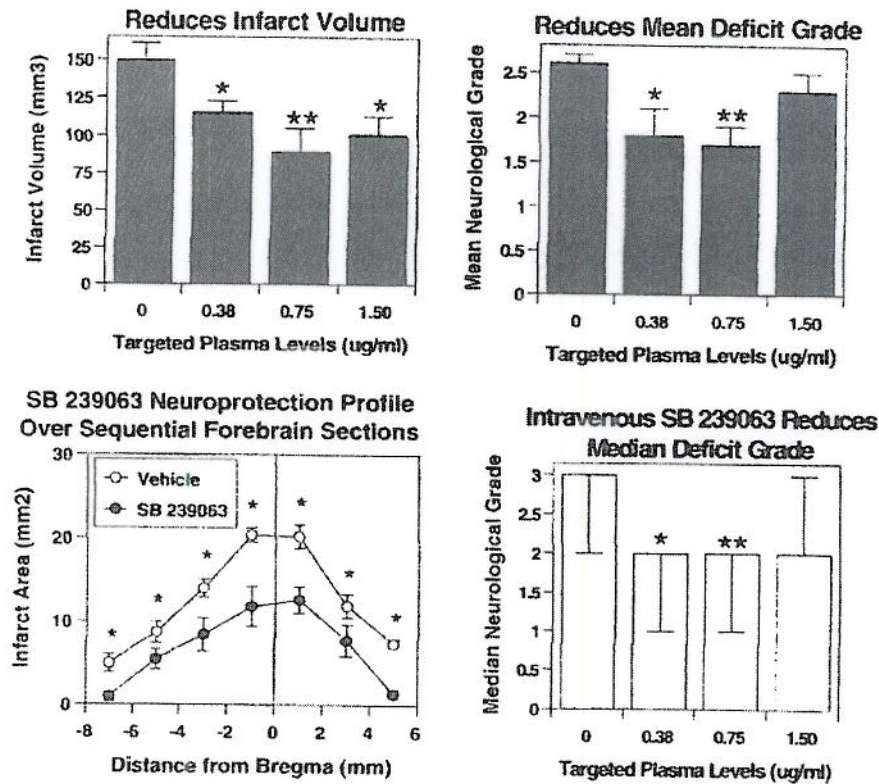


Fig. 3. Neuroprotective effects of SB 239063 in moderate stroke (SHR MCAO cauterization at level of inferior cerebral vein). Significant reductions in total infarct volume (top left), profile of infarct areas on individual forebrain slices for vehicle versus 0.75 $\mu\text{g}/\text{ml}$ SB 239063 targeted plasma level (bottom left), and neurological deficits presented as means (top right) or medians (bottom right). $n = 7-16$ per group; * $p < 0.05$ different from vehicle group; ** $p < 0.01$ different from vehicle group.

strate the efficacy of SB 239063. These models, which differ in respect to rat strain, ischemic severity, anesthesia, and in-dependent sites/laboratory locations, have been well validated in many neuroprotection studies (Mackay et al., 1996; Wood et al., 1997; Spera et al., 1998; Chandra et al., 1999; Campbell et al., 2000). Therefore, the robust neuroprotection of SB 239063 has been demonstrated by the efficacy exhibited in these different models of permanent ischemic stroke. We have found that neuroprotection using a variety of protective agents is much more difficult to demonstrate in permanent stroke models (i.e., as compared with transient focal ischemia with reperfusion). In both of these permanent stroke models, neuroprotection with intravenous SB 239063 occurred at plasma concentrations that were expected to provide potent p38 inhibition. The methods for tissue injury analyses used in these two stroke models have been cross-validated between laboratory sites and with magnetic resonance imaging measurements previously (i.e., identical measures are obtained with these procedures). It is important to mention that we have also extended these studies to longer periods of permanent focal stroke and have monitored protection (i.e., reduced infarct size due to SB 239063 treatment) over a 1-week period using magnetic resonance imaging of brain injury (data not shown).

The rapid phosphorylation of p38 following stroke (Irving et al., 2000) suggested that the activation of this signaling cascade may be, to some degree, independent of the brain inflammatory response. We therefore evaluated the effects of SB 239063 in a model of OGD-induced cell death. The compound also demonstrated direct neuroprotective activity in

this isolated cell-based brain ischemia model. This suggests that this second-generation p38 inhibitor can protect neurons directly in addition to effects at blocking inflammatory cytokine/mediator production and subsequent brain inflammation.

The in vivo U-shaped dose-response curve is not specific to p38 inhibition-induced brain protection but occurs for other classes of neuroprotective compounds as dose administrations are increased (Tatlisumak et al., 1998; Takahashi et al., 1999). This lack of efficacy as doses are escalated is apparently related to loss of drug selectivity at higher concentrations (i.e., caused by nonspecific effects of the high concentration of the drug at other targets). This U-function was exhibited both in vitro and in vivo in the present studies, suggesting that more direct cellular aspects of the molecule are involved in this protective function. The fact that it is active in vitro and that no significant effects of this class of compounds on many parameters (i.e., blood pressure, heart rate, cerebral perfusion, or body/brain temperature; blood glucose or blood gases, data not shown) have been observed suggests that its protection is due primarily to inhibition of p38. The SB 239063 data presented in Table 4 further substantiates this interpretation.

Increased interleukin-1 β and TNF α message and protein in neurons, astrocytes, and microglia following stroke has been documented in many studies (Barone, 1998; Davies et al., 1998; Barone and Feuerstein, 1999; Pearson et al., 1999; Currie et al., 2000). The present and our recent previous data (Irving et al., 2000) demonstrate that p38 activation occurs in neurons and glial cells and that this activation apparently

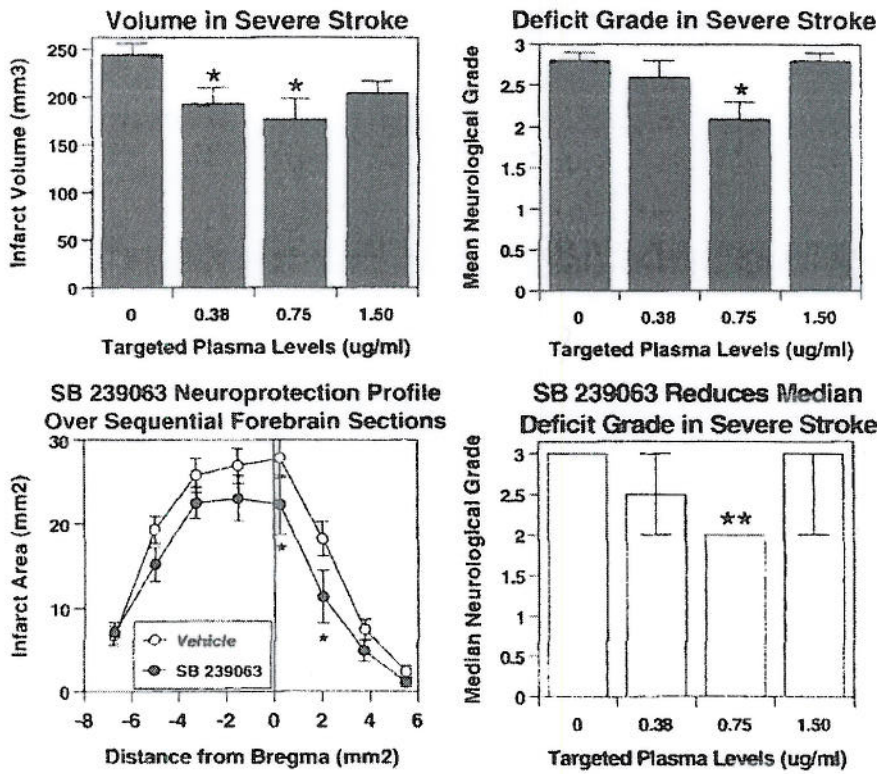


Fig. 4. Neuroprotective effects of SB 239063 in severe stroke (TNR MCAO using suture at MCA origin from circle of Willis). Significant reductions in total infarct volume (top left), profile of infarct areas on individual forebrain slices for vehicle versus 0.75 μ g/ml SB 239063 targeted plasma level (bottom left), and neurological deficits presented as means (top right) or medians (bottom right). $n = 14-15$ per group; * $p < 0.05$ different from vehicle group; ** $p < 0.01$ different from vehicle group.

TABLE 4
Effects of SB 239063 on cortical perfusion, hemodynamics, and temperature*

	Absolute Values				Percentage Change from Baseline			
	Vehicle		SB 239063		Vehicle		SB 239063	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
MABP								
Baseline	141.3	3.2	141.0	8.3	0.0	0.0	0.0	0.0
20 min	144.3	3.7	139.6	6.9	2.1	0.7	-0.7	1.4
40 min	141.2	3.3	141.6	8.2	0.0	1.7	0.6	2.3
60 min	140.8	4.1	145.2	8.2	-0.2	3.2	3.3	3.0
HR								
Baseline	357.5	14.9	365.0	14.5	0.0	0.0	0.0	0.0
20 min	354.2	15.0	351.0	14.5	-0.9	0.3	-3.9	0.7
40 min	351.7	13.7	342.0	14.2	-1.6	0.6	-6.3	0.8
60 min	345.0	11.3	337.0	11.5	-3.3	1.5	-7.5	1.9
LDF								
Baseline	204.0	5.8	202.0	4.4	0.0	0.0	0.0	0.0
20 min	196.0	9.4	196.0	4.3	-4.0	2.7	-3.0	0.5
40 min	194.0	9.3	194.0	5.3	-5.0	2.9	-4.0	1.0
60 min	191.0	9.9	190.0	4.7	-6.4	3.6	-5.9	1.2
BT								
Baseline	36.9	0.2	36.9	0.2	0.0	0.0	0.0	0.0
20 min	36.7	0.2	36.8	0.1	-0.5	0.3	-0.4	0.2
40 min	36.7	0.3	36.7	0.2	-0.6	0.3	-0.6	0.2
60 min	36.7	0.2	36.6	0.2	-0.5	0.3	-0.9	0.3

* Measurements carried out under experimental conditions as described under Materials and Methods.

participates in the induction of these inflammatory cytokines and apoptosis/cell death. It is interesting that this activation occurs in all areas destined to be infarcted; in the developing infarct (i.e., within astrocytes), in the peri-infarct area (i.e., within neurons), and in the subcortical white matter (i.e., within all types of glial cells) (Irving et al., 2000). The con-

vergence/importance of inflammatory cytokines and apoptotic pathways has been demonstrated previously (Hara et al., 1997; Sidoti-de Fraisse et al., 1998). In addition, other cytotdestructive enzymes (e.g., inducible nitric-oxide synthase and cyclooxygenase II) induced through the p38 pathway can contribute significantly to stroke-induced brain injury (Bhat

SB 239063 Reduces Neuronal Cell Death In Vitro

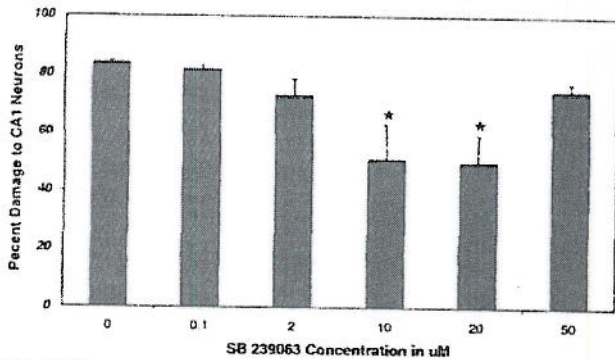


Fig. 5. Direct neuroprotective effects of SB 239063 on OGD in organotypic hippocampal brain slices. Percent damage to CA1 neurons in hippocampal neurons in brain slices 23 h following 45 min of OGD (in vitro ischemia) was significantly reduced by SB 239063. $n = 9$ per concentration bar. * $p < 0.05$ different from zero concentration control group.

et al., 1998; Ridley et al., 1998). The advantage of combined therapeutic approaches (i.e., reducing apoptosis, cytotoxic mediators, and the production of inflammatory cytokines/inflammation) inherent to p38 inhibition following focal stroke is obvious.

Additional studies will be required to fully understand and characterize the protective effects of SB 239063. However, the present data is the first demonstration of significant in vivo neuroprotection and associated improved functional outcome that can occur due to the poststroke administration of a second-generation p38 inhibitor, SB 239063. These data suggest that p38 is involved in the progression of cell death in focal ischemia. These and other recent studies (Alessandrini et al., 1999) strongly support the importance of MAPK signaling in brain injury and that a significant opportunity might be provided by learning more about MAPK signaling and its role in brain injury. This approach should be extended in the future by evaluating the inhibition of MAPKs in additional models of central nervous system injury, including cerebral hemorrhage and brain trauma (Barone, 1998), and also in spinal cord injury (Nakahara et al., 1999) and neurodegenerative diseases (Hensley et al., 1999).

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