To determine whether BSF468248 is effective against focal cerebral ischemia, we evaluated the change of cerebral blood flow (CBF) and infarction volume using a photochemically-induced cortical vein occlusion model in rats in blind studies. In 22 Wistar rats, two adjacent cortical veins were occluded by photochemical thrombosis and fiberoptic illumination under controlled anesthesia and ventilation. Just after the occlusion, BSF468248 or physiological saline was administrated. In the low-dose study, a treatment group (n = 7) was administered BSF468248 1 mg/kg bolus and 1 mg/kg continuously for 30 min. The same volume of saline was given to a vehicle group (n = 5). In the high-dose study, a treatment group (n = 5) was administrated BSF468248 1 mg/kg bolus and 12 mg/kg continuously for 180 min. The same volume of saline was given to a vehicle group (n = 5). During the experiment, regional cerebral blood flow (rCBF) was measured in both the low-dose study (120 min) and the high-dose study (180 min). Seven days after the experiment, the animals were killed in order to evaluate the infarct volume. The rCBF at the end of the experiment showed a similar decrease in both the low-dose study (at 120 min: treatment group: 66.5 ± 10.2%; vehicle group: 69.3 ± 10.2%) and the high-dose study (at 180 min: treatment group: 62.1 ± 7.5%; vehicle group: 65.1 ± 12.3%), with no significant differences (t-test). The infarct volume also showed no significant difference in either group of the low-dose study (treatment group: 3.46 ± 0.84 mm^3; vehicle group: 3.56 ± 1.40 mm^3) or the high-dose study (treatment group: 2.27 ± 0.43 mm^3; vehicle group: 1.76 ± 0.31 mm^3). Our study found that BSF468248 is not effective in improving the rCBF and the infarct volume following focal cerebral ischemia.
intubated with silicon tube (o.d. 2.5 mm), and animals were mechanically ventilated with 30% oxygen using a rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA, USA) under control of end expiratory PCO₂ (Artema MM206C; Heyer, Sweden). Rectal temperature was kept close to 37.0 °C throughout the experiment by a feedback-controlled heating pad (Harvard Apparatus). Polyethylene catheters (o.d. 0.96 mm, Portex) were inserted into the right femoral artery and vein. The arterial line served for continuous registration of mean arterial blood pressure (MABP) and arterial blood gas sampling and the venous lined for administration of fluid and drugs. PaO₂, PaCO₂, arterial pH, electrolytes, glucose and lactate were measured with a blood gas and electrolyte analyzer (ABL System 615; Radiometer, Copenhagen, Denmark). Blood pressure was continuously connected to a pressure transducer (Datascopy 870 Monitor & Pressure Display Module; Datascopy Corp., Paramus, NJ, USA). Rats were mounted in a stereotaxic frame (Sotelting, Wood Dale, IL, USA). After a 2.0-cm midline skin incision, a right frontal-parietal cranial window was made for access to the brain surface using a high-speed drill under an operating microscope (OP-Microscope, Zeiss, Wetslar, Germany). During the craniectomy, the drill tip was cooled continuously with physiological saline to avoid thermal injury to the cortex.

After exposition of dura mater, our original heating reflector, which was connected with a controllable direct-current transformer (dual-output power supply EA-3023, Elektro-Automatik, Viersen, Germany), was used for peri-ischemic temperature control close to 36.0 °C with measurement of temperature of right temporal muscle (Oximetrix 3; Abbott, Chicago, IL, USA) (6).

**Cortical vein occlusion by photochemical thrombosis**

The protocol for cortical vein occlusion by photochemical thrombosis has been previously described in detail (7, 8). The occlusion of two adjacent cortical veins was induced using rose bengal dye (Sigma, St. Louis, MO, USA) and fiberoptic illumination using a 50-W mercury lamp (6500-7500 lx, 540 nm) connected to a 100-µm fiber through the lucid dura mater. Only animals with a similar venous anatomy were used, i.e., with two prominent adjacent veins connecting into the superior sagittal sinus, and the distance between the two veins was about 2.0-3.0 mm. The diameter of the occluded veins was approximately 100 µm. Rose bengal (50 mg/kg BW) was injected slowly, without effecting the systemic arterial pressure, and care was taken to avoid illumination of tissue and other vessels near the target vein. The vein was illuminated for 10 min via the micro-manipulator-assisted light guide. To occlude the second selected vein, half of the initial rose bengal dose was injected intravenously before the illumination was repeated with the new target.

**Measurement of CBF using LD flowmetry**

Local CBF was measured by laser Doppler (LD) (Model BPM 403a; Vasomedics, St. Paul, MN, USA) with 0.8-mm needle probes. Flow is expressed in LD units, which are not arbitrary but have a low biological zero (0-1 LD units) and are one-point calibrated with latex beads at 25 °C in a Teflon vial. We have previously shown that LD readings correlate linearly with hydrogen clearance provided that several adjacent cortical locations are scanned by LD (9) to overcome the high spatial variability of local flow measurements.

Local CBF was measured at 36 (6 x 6) locations each at a distance of 500 µm with the occluded veins lateral to the scanning field (2.5 x 2.5 mm) using a stepping-motor-driven and computer-controlled micromanipulator (7, 8, 10). The median of the 36 local CBF values from individual rats is termed regional CBF (rCBF). The technique permits repeated scans for a given set of locations. Scanning was performed from beginning to the end of the experiment at identical locations at 15-min intervals.

**Experimental protocol**

Two blind tests were carried out for different dosages of the drug. The BSF468248 solution is colorless, lucid and odorless.

In the low-dose study, intravenous bolus of BSF468248 (BASF pharma, Ludwigshafen, Germany; 1 mg/g BW, vehicle group, n = 7) or the same volume of physiological saline (vehicle group, n = 5) was injected just after occlusion of cortical veins. Moreover, the same volume of the drug (treatment group) or saline (vehicle group) was infused continuously following 30 min with a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden). The follow up was for 120 min after venous occlusion.

In the high-dose study, the drug (1 mg/g b.w., treatment group, n = 5) or the same volume of saline (vehicle group, n = 5) was injected in a bolus fashion, as in the low-dose study. Then the high-dose drug (4 mg/g b.w., treatment group) or the same volume of saline (vehicle group) was infused continuously for following 180 min, namely the volume of 12 mg/g b.w. for continuous infusion.

**Histological preparation**

After the operation, skin wounds were closed with silk thread. The rats were returned to individual cages and allowed free access to water and food. The rats were perfusion-fixed with 4% paraformaldehyde after general anesthesia with chloral hydrate. Then, the brain was carefully removed from the skull. Brains were embedded in paraffin to obtain coronal sections (3 µm) of the parietal regions. Sections were stained with hematoxylin/eosin and luxol fast blue. The histological evaluation was performed by light microscopy. A CCD camera (Sony, Tokyo, Japan) and a Maxigen Genelock interface
RESULTS

Physiological variables
Under control conditions the physiological variables, such as blood gases (PaO\textsubscript{2}, PaCO\textsubscript{2} and pH), electrolyte, glucose, lactate, mean arterial blood pressure, body temperature and temporal-muscle temperature, showed no significant differences and were within the physiological range between groups in both blind tests (Table 1; Figure 1A and 1B).

Regional cerebral blood flow
Regional cerebral blood flow (rCBF) showed gradual decrease after venous occlusion (Figure 1A and 1B).

TABLE 1. Data from arterial blood gas analyses sampled during control conditions and after the follow-up time.

<table>
<thead>
<tr>
<th></th>
<th>PH</th>
<th>PaO\textsubscript{2} (mmHg)</th>
<th>PaCO\textsubscript{2} (mmHg)</th>
<th>Hemoglobin (g/dl)</th>
<th>Hematocrit (%)</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
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<tr>
<td>Low-dose study</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment (n = 5)</td>
<td>7.47 ± 0.01</td>
<td>7.39 ± 0.01</td>
<td>141.3 ± 10.5</td>
<td>142.2 ± 13.4</td>
<td>38.2 ± 0.6</td>
</tr>
<tr>
<td>Vehicle (n = 5)</td>
<td>7.45 ± 0.00</td>
<td>7.37 ± 0.02</td>
<td>135.5 ± 6.8</td>
<td>144.5 ± 12.7</td>
<td>40.0 ± 0.4</td>
</tr>
<tr>
<td>High-dose study</td>
<td></td>
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<tr>
<td>Treatment (n = 5)</td>
<td>7.48 ± 0.02</td>
<td>7.37 ± 0.01</td>
<td>139.4 ± 13.0</td>
<td>156.5 ± 10.2</td>
<td>36.9 ± 0.8</td>
</tr>
<tr>
<td>Vehicle (n = 5)</td>
<td>7.49 ± 0.01</td>
<td>7.40 ± 0.02</td>
<td>153.8 ± 8.0</td>
<td>158.0 ± 8.0</td>
<td>35.9 ± 1.1</td>
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<table>
<thead>
<tr>
<th></th>
<th>Na\textsuperscript{+} (mmol/l)</th>
<th>K\textsuperscript{+} (mmol/l)</th>
<th>Glucose (mg/dl)</th>
<th>Lactate (mmol/l)</th>
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<td>Before</td>
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<tr>
<td>Low-dose study</td>
<td></td>
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</tr>
<tr>
<td>Treatment (n = 7)</td>
<td>132.5 ± 1.2</td>
<td>131.5 ± 0.5</td>
<td>5.3 ± 1.2</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Vehicle (n = 5)</td>
<td>131.5 ± 1.5</td>
<td>131.0 ± 3.0</td>
<td>5.2 ± 0.2</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>High-dose study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment (n = 5)</td>
<td>132.0 ± 1.2</td>
<td>130.1 ± 0.8</td>
<td>5.2 ± 0.2</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Vehicle (n = 5)</td>
<td>131.6 ± 0.5</td>
<td>131.0 ± 0.5</td>
<td>4.8 ± 0.1</td>
<td>4.0 ± 0.1</td>
</tr>
</tbody>
</table>

Values are mean ± standard error of mean. There are no statistical differences between the two groups in both the low-dose and high-dose study.
ischemic injury in both the treatment group and the vehicle group of the high-dose study.

Complement activation in cerebral ischemia

Previous studies have reported on whether complement activation contributes to brain damage after cerebral ischemia. Many studies have shown the relationship between complement activation and cerebral ischemia. A previous investigation of human brain following fatal ischemic brain infarction showed complement activation

BSF468248 and C1-INH were 0.01 and 0.2, respectively, in rats (unpublished data). We studied this protective effect against focal cerebral ischemia in two blind tests using both a low dose (bolus 1 mg/kg b.w., infusion 1 mg/kg b.w.) and a high dose (bolus 1 mg/kg b.w., infusion 12 mg/kg b.w.). In spite of the potent effect in the enzyme test, this drug did not improve the infarct volume in the present study.

The infarct volume of the high-dose study tended to be smaller than that of the low-dose study. This may be due to the longer time of anesthesia that protected against ischemic injury in both the treatment group and the vehicle group of the high-dose study.

Complement activation in cerebral ischemia

Previous studies have reported on whether complement activation contributes to brain damage after cerebral ischemia.

FIG. 1. Time course of regional cerebral blood flow (rCBF) measured by laser Doppler scanning in the treatment group and vehicle group in the low-dose study (A) and high-dose study (B). The photothrombosis of two cortical veins induced a critical flow reduction. However, there was no difference in rCBF or mean arterial blood pressure (MABP) (inset) between the two groups.
Moreover, Huang et al. (12) also reported that covalently modified sCR1 by sialyl Lewis x (sLe\text{x}) glycosylation (sCR1sLe\text{x}) reduced cerebral infarction volume in transient MCA occlusion model in mouse. This hybrid molecule inhibits both complement activation and selectin-mediated adhesion. CR1 has not only the effects stated above but also the effect as a ligand for C1q (14). In the study, ischemic neurons expressed C1q and sCR1 bound these neurons. Furthermore, the sLe\text{x} moiety binds cell surface selectins and blocks selectin-mediated cellular adhesion (15). Anaphylatoxin C3a and C5a receptors (C3aR and C5aR) expressed at the mRNA and protein levels in ischemic brain tissues following permanent middle cerebral artery occlusion in the mouse (16). The protein levels of C3aR and C5aR were found constitutively on neurons and astrocytes. After ischemia, they were increased in macrophage-like cells and astrocytes and expressed de novo on endothelial cells. The mRNA levels were also increased and reached a peak at 2 days after MCA occlusion. C3aR and C5aR can also activate cells to produce cytokines, neurotrophins, acute phase proteins, and express increased level of adhesion molecules (16).

On the other hand, the recent previous study showed that complement depletion using cobra venom factor in a rabbit model of stroke, with and without subsequent tissue plasminogen activator thrombolysis, had not significantly affected the degree of ischemic cerebral injury (17). In that study, the rCBF, the infarct volume, and intracranial pressure after cerebral ischemia were not affected by the pretreatment of the depletion of complement. The authors suggest that complement activation may not be a significant mediator of acute stroke injury.

The relation between complement activation and cerebral ischemia remains controversial.

**Possible reasons of invalidity**

There are several possible reasons why BSF468248 is not effective against focal cerebral ischemia.

Firstly, there may be little relation between complement activation and cerebral ischemia. However, as described above, many articles showed data that suggested this relationship. From the results of the present study, it is impossible to say that there is no relationship between complement activation and cerebral ischemia.

Secondly, this drug may have not reached the brain parenchyma. The half-life for existence in the circulation may be shorter than that of the C1-INH, which is approximately 64 h (18). Because this molecule is an oligopeptide and its molecular weight of BSF468248 is only 520.05, it may be catabolized and excreted immediately before arrival into the ischemic brain. However, this drug may have little ability to penetrate the blood-brain barrier (BBB). The ability of a particular solute to cross the BBB by diffusion is largely dependent on two related factors; lipophilicity and hydrogen bonding potential, but not molecular weight (19). The higher the lipophilicity or the lower the hydrogen bonding potential, the higher the diffusion into the brain. However, breakdown of the BBB in the acute stage after cerebral infarction has been reported (20, 21). As mentioned above, the C1-INH is effective against focal cerebral ischemia. The long half-life of the C1-INH may enable penetration of the BBB at the time of breakdown following cerebral ischemia.

Thirdly, the use of the drug may not be appropriate to obtain the effect against focal cerebral ischemia. BSF468248 was administrated intravenously in the present study and differed from the C1-INH that was given intraterially in the previous study (4). The first-pass effect may affect the catabolism of this drug at the liver and sufficient concentration or volume for validity may not be achieved. Moreover, the administrated dose may be low in order to improve the brain tissue damage. Even in the treatment group of the high-dose study no adverse effect appeared in the physiological data.
Experimental model

The photochemically-induced two-vein occlusion model (7, 8) was used in the present study. It is possible to keep the dura mater intact because of its lucidity. LD scanning flowmetry (10) was used to measure the rCBF. Ischemic tissue damage can be made without direct manipulations on the brain surface such as needle insertions and direct thermal damage during the experiment. These factors result in high reliability and reproducibility using this model.

CONCLUSION

In conclusion, our study found that BSF468248 is not effective in improving the rCBF and the infarct volume following focal cerebral ischemia induced by photochemical venous occlusion using rats.

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REFERENCES


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