Cilostazol Ameliorates Warfarin-Induced Hemorrhagic Transformation After Cerebral Ischemia in Mice

Akira Kitashoji, BS; Yusuke Egashira, MD, PhD; Keisuke Mishiro, BS, RPh; Yukiya Suzuki, BS, RPh; Hideki Ito, PhD; Kazuhiro Tsuruma, PhD, RPh; Masamitsu Shimazawa, PhD, RPh; Hideaki Hara, PhD, RPh

Background and Purpose—Although long-term treatment with the oral anticoagulant warfarin is widely used to prevent cardioembolic ischemic stroke, it has been reported that warfarin can exacerbate hemorrhagic transformation (HT) after cerebral ischemia. We investigated whether cilostazol, a phosphodiesterase-III inhibitor, suppressed the warfarin-induced HT after cerebral ischemia in mice.

Methods—Male ddY mice were treated with oral warfarin before 3-hour middle cerebral artery occlusion followed by 21-hour reperfusion to induce HT. The duration of warfarin pretreatment was determined by measurement of prothrombin time-international normalized ratio value. Cilostazol or vehicle was administered by intraperitoneal injection immediately after reperfusion. The infarct volume, brain swelling, and brain hemoglobin content were evaluated at 24 hours after middle cerebral artery occlusion. We also evaluated the survival rate of each treated group for 7 days after surgery. To investigate the mechanism underlying cilostazol’s effects, the proteins involved in vascular endothelial integrity were investigated using Western blotting.

Results—HT volume was exacerbated by warfarin treatment, and cilostazol (3 mg/kg, IP) suppressed this exacerbation (sham, mean±SD, 29.2±13.4 mg/dL; vehicle, 33.3±11.9 mg/dL; warfarin, 379.4±428.9 mg/dL; warfarin+cilostazol 1 mg/kg, 167.5±114.2 mg/dL; warfarin+cilostazol 3 mg/kg, 116.9±52.3 mg/dL). Furthermore, cilostazol improved survival rate and upregulated the expression of tight junction proteins and vascular endothelial cadherin.

Conclusions—Cilostazol reduced the warfarin-related risk of HT after ischemia by protecting the vascular endothelial cells. This result suggested that cilostazol administration in patients with acute ischemic stroke might reduce HT. (Stroke. 2013;44:00-00.)

Key Words: anticoagulants ◼ cilostazol ◼ hemorrhage ◼ mice ◼ stroke ◼ warfarin

Stroke is strongly related to age. With the increase in aging populations in developed countries, the number of patients with cardioembolic ischemic stroke resulting from nonvalvular atrial fibrillation, the major cause of cardioembolic ischemic stroke, is predicted to double by 2030.1,2 Administration of oral anticoagulants has been widely accepted and strongly recommended as the standard treatment for the prevention of cardioembolic ischemic stroke caused by nonvalvular atrial fibrillation,3 especially in patients with high-risk comorbidities such as hypertension, diabetes mellitus, congestive heart failure, or a previous ischemic stroke.4

Warfarin, a vitamin K antagonist, is the most widely used oral anticoagulant worldwide for the prevention of cardioembolic ischemic stroke in patients with nonvalvular atrial fibrillation. Although other novel oral anticoagulants, namely, dabigatran and rivaroxaban, are associated with relatively lower risks of the hemorrhagic complications than is warfarin,6 the lack of specific antagonists is a major concern associated with their clinical use.7 Therefore, warfarin continues to be the standard oral anticoagulant in clinical practice. Early hemorrhagic transformation (HT) can occur as a complication of ischemic stroke. The size of the ischemic lesion and cardioembolic ischemic stroke were determined as independent risk factors of early HT after ischemia.8 Moreover, parenchymal hematoma, a severe subtype of early HT after cerebral ischemia, was independently associated with adverse patient outcomes.8 However, no effective treatment strategy is available for prevention of HT in clinical practice. Experimental studies of cerebral ischemia have established increase in the permeability of the blood–brain barrier (BBB) after ischemia/reperfusion injury as one of the major causes of HT.9,10 Although few studies have been published on HT risk after cerebral ischemia in patients receiving oral warfarin, pretreatment with warfarin drastically
increased the risk of HT after transient focal cerebral ischemia in a mouse model.\textsuperscript{11}

Cilostazol is an antiplatelet agent that acts via inhibition of phosphodiesterase-III. It is widely used for secondary prevention of cerebral infarction and treatment of intermittent claudication in peripheral artery disease. One of the characteristic features of cilostazol is its potential to protect the vascular endothelium.\textsuperscript{10,12–14} We previously demonstrated that cilostazol reduced HT after cerebral ischemia, via prevention of BBB disruption in mice.\textsuperscript{10,12}

In the present study, we extend this work to clarify the efficacy of cilostazol in preventing warfarin-induced HT after cerebral ischemia in a mouse model.

**Methods**

**Animals**

All animal protocols were conducted in accordance with the Animal Research: Reporting In Vivo Experiments guidelines and were approved by the animal experiment committees of Gifu Pharmaceutical University, Japan. All experiments were performed using male ddY mice (4 weeks old; body weight, 20–30 g; Japan SLC Ltd, Hamamatsu, Japan). Animals were housed at 24°C±2°C under a 12-hour light–dark cycle. Food and water were available to all animals ad libitum. The operators (A. Kitashoji, K. Mishiro, and Y. Suzuki) were blinded to the treatment status of the animals in all experiments.

Warfarin (5 g; 0.2% warfarin K fine granules; Youshindou, Toyama, Japan) was dissolved in 375 mL of water, and the mice were fed for 0, 6, 12, 18, or 24 hours with free access to the treated water. Assuming a mouse body weight of 20 g and a water consumption rate of 15 mL/100 g 6, 12, 18, or 24 hours with free access to the treated water, the warfarin concentration in the treated water was 0.17 mg/mL.

Experimental Procedures

The operator was blinded to the treatment status of the animals. To induce focal cerebral ischemia, the mice were anesthetized with 2.0% to 2.5% isoflurane (Mylan Inc, Canonsburg, PA) by using a facemask (The Univentor 400 Anesthesia Unit, Bioresearch Center Co Ltd, Nagoya, Japan) 18 hours after administration of warfarin. Middle cerebral artery occlusion (MCAO) was induced by insertion of a silicone-coated 8-0 nylon monofilament (Ethicon, Somerville, NJ), as described previously.\textsuperscript{15} Three hours after this procedure, the mice were briefly re-anesthetized with isoflurane, and the middle cerebral artery blood flow was restored by withdrawing the nylon monofilament. Sham-control mice underwent the same surgical procedure but without middle cerebral artery obstruction. Immediately after reperfusion, a dose of 1 or 3 mg/kg of cilostazol (Otsuka Pharmaceutical Co Ltd, Tokushima, Japan) in 0.5% carboxymethyl cellulose was injected intraperitoneally by using a 1-mL syringe and a 26-gauge needle. The injection volume was set at 8 mL/kg body weight. The vehicle-treated control mice were injected with the same volume of 0.5% carboxymethyl cellulose. Mouse body temperature was maintained at 37°C to 37.5°C with the aid of a heating pad and heating lamp during all surgeries.

We used a total of 327 mice in this study and excluded 21 mice based on the following criteria for exclusion, which we set before the experiments: animals for which prothrombin time-international normalized ratio (PT-INR) values could not be measured, that died by excessive anesthesia or procedural problems that occurred during operations, and that died up to sampling after operations. Furthermore, as failures of MCAO/reperfusion, animals that were withdrawn or has been escaped the nylon monofilament at the time of reperfusion were excluded.

The online-only Data Supplement provides further details of group sizes, the methods used for measurement of PT-INR, measurement of infarct volume, spectrophotometric hemoglobin assay, evaluation of survival rate, evaluation of matrix metalloproteinases (MMPs) 2 and 9, tight junction proteins, and vascular endothelial (VE) cadherin expression, measurement of bleeding time, and statistical analyses.

**Results**

**PT-INR Value**

The PT-INR values after warfarin administration increased in a time-dependent manner (PT-INR: 0 hour, 0.73±0.17; 6 hours, 0.90±0.17; 12 hours, 1.32±0.49; 18 hours, 3.87±1.10; 24 hours, 5.75±1.52; Figure 1) and were higher than those previously reported.\textsuperscript{15} This may be because of differences between strains of mice. We carried out all subsequent experiments using warfarin administration for 18 hours because the PT-INR value at 24 hours was high but this value rose between 18 and 24 hours. (At the time of planning: 0 hour, n=10; 6 hours, n=10; 12 hours, n=10; 18 hours, n=10; 24 hours, n=10. Final: 0 hour, n=6; 6 hours, n=7; 12 hours, n=8; 18 hours, n=10; 24 hours, n=10. Nine animals for which PT-INR values could not be measured because of procedural problems were excluded.)

**Effects of Warfarin and Cilostazol on Infarct Volume and Swelling**

We evaluated the infarct volume and swelling 21 hours after reperfusion. (At the time of planning: vehicle, n=15; warfarin, n=15; warfarin+cilostazol 1 mg/kg, n=10; warfarin+cilostazol 3 mg/kg, n=15. Final: vehicle, n=15; warfarin, n=15; warfarin+cilostazol 1 mg/kg, n=7; warfarin+cilostazol 3 mg/kg, n=13. Five animals were excluded: in warfarin+cilostazol 1 mg/kg group, 2 mice died at the time of reperfusion and 1 mouse died before sampling; and in warfarin+cilostazol 3 mg/kg group, 1 mouse died just after reperfusion and 1 mouse died before sampling.) Infarct size and volume were not significantly different between the groups (Figure 2A–2C). However, compared with the vehicle group, the group that received warfarin showed significantly exacerbated swelling. However, cilostazol (3 mg/kg) suppressed this exacerbation (Figure 2D).

**Effects of Warfarin and Cilostazol on Hemorrhagic Transformation**

We next evaluated the hemorrhage volume in ischemic brains by measuring the hemoglobin content of extravasated blood.

![Figure 1. Prothrombin time-international normalized ratio values in mice after 0, 6, 12, 18, and 24 hours of warfarin administration in drinking water. PT-INR indicates prothrombin time-international normalized ratio. Data are expressed as mean±SD. **P<0.01 vs 0 hour; Dunnett t test, 0 hour, n=8; 6 hours, n=7; 12 hours, n=8; 18 hours, n=10; 24 hours, n=10.](image)
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Effects of Warfarin and Cilostazol on Survival Rate After Ischemia and Reperfusion
After MCAO, mouse body weights gradually decreased until day 4 in the warfarin monotherapy group and until day 2 in the combination group. (At the time of planning: warfarin, n=12; warfarin+cilostazol 3 mg/kg, n=12. Final: warfarin, n=12; warfarin+cilostazol 3 mg/kg, n=10. Two animals were excluded: in warfarin+cilostazol 3 mg/kg group, 1 mouse died before reperfusion and another at the time of reperfusion.) Body weight tended to decrease more in the warfarin monotherapy group than in the combination group but then recovered and increased by 7 days after MCAO in the both groups (data not shown). At this time point, the survival rate of the combination group was significantly higher than that of the warfarin monotherapy group (final survival rates 80% and 33.3%, respectively; P=0.0494; log-rank t test; Figure 3D).

Effects of Warfarin and Cilostazol on MMP-2 and MMP-9 Expression
We investigated MMP-2 and MMP-9 protein expression levels. (At the time of planning: sham, n=6; vehicle, n=15; warfarin, n=15; warfarin+cilostazol 3 mg/kg, n=15. Final: sham, n=6; vehicle, n=14; warfarin, n=11; warfarin+cilostazol 3 mg/kg, n=14. Six animals were excluded: in vehicle group, 1 mouse died before sampling; in warfarin group, 3 mice died because the common carotid artery was cut during operations, before reperfusion, and at the time of reperfusion; and in 1 mouse the nylon monofilament could not be withdrawn at the time of reperfusion; and in warfarin+cilostazol 3 mg/kg group, 1 mouse died before sampling.) Only MMP-9 expression level increased because of ischemia; however, neither warfarin nor cilostazol affected MMP-2 or MMP-9 expression levels (Figure 1 in the online-only Data Supplement).

Effects of Warfarin and Cilostazol on Tight Junction Proteins and VE-Cadherin Expression
We investigated the effects of each therapy on tight junction proteins and VE-cadherin expression levels. (At the time of planning: sham, n=10; vehicle, n=10; warfarin, n=10; warfarin+cilostazol 3 mg/kg, n=10. Final: sham, n=9; vehicle, n=9; warfarin, n=8; warfarin+cilostazol 3 mg/kg, n=8. Five animals were excluded: in sham group, 1 mouse died by excessive anesthesia; in vehicle group, 1 mouse died just after reperfusion; in warfarin group, 1 mouse died before reperfusion; and in warfarin+cilostazol 3 mg/kg group; 2 mice died before reperfusion.) Occludin levels were not changed because of ischemia or because of warfarin or cilostazol administration (Figure 4B). However, ZO-1, claudin-5, and VE-cadherin levels significantly decreased because of ischemia and increased

Figure 2. Effects of warfarin and CSZ on cerebral infarction and swelling in mice at 21 hours after reperfusion. Triphenyltetrazolium chloride staining of coronal sections (2-mm thick) at 21 hours after reperfusion in representative mice (A). These figures show the effects of warfarin and CSZ on the cerebral infarct area (B), volume (C), and swelling (D). CSZ indicates cilostazol; and W, warfarin. Data are expressed as mean±SEM. *P<0.05 vs vehicle; **P<0.01 vs warfarin; Tukey t test, vehicle, n=15; warfarin, n=15; warfarin+CSZ 1 mg/kg, n=7; warfarin+CSZ 3 mg/kg, n=13.
because of the administration of cilostazol. However, no significant differences were noted between the vehicle group and the warfarin administration group (Figure 4A, 4C, and 4D).

**Effects of Warfarin and Cilostazol on Tail Vein Bleeding Time**

The bleeding time was significantly prolonged by warfarin administration but not by the combination of cilostazol (3 mg/kg, IP) and warfarin (normal, mean±SD, 251.3±64.0 seconds; warfarin, 596.3±149.9 seconds; warfarin+cilostazol 3 mg/kg, 600.0±135.1 seconds) (n=8 animals; Table 1).

**Discussion**

HT occurs after ischemic stroke, and it is considered that a main mechanism of HT is the leakage of blood by the disruption of the BBB; however, there is no definite consensus. It has been reported that HT is a marker of early reperfusion, reduced infarct size, and hence, improved functional outcome, whereas parenchymal hematoma is a marker of delayed reperfusion and poor outcome. Contrary to this report, it has been reported that HT (small petechiae) is not a predictor of outcome, and that hemorrhagic infarction (confluent petechiae) and parenchymal hematoma are negative predictors of outcome. This hypothesis is "no hemorrhage is likely to be associated with the best outcome." This report concluded that asymptomatic hemorrhage after thrombolysis may not be benign. Accordingly, mild HT means early recanalization and may predict better outcome, whereas serious HT is linked to a poor prognosis and exacerbated neurological symptoms after ischemic stroke.

However, despite its clinical significance, no effective treatment for HT has been identified.

Although warfarin greatly reduces the risk of cardioembolic ischemic stroke, many patients receiving warfarin for atrial fibrillation experience a stroke at some stage in their life. In previous studies of the effects of anticoagulants on stroke severity in patients with atrial fibrillation, ~30% of patients with cardioembolic ischemic strokes received warfarin anticoagulation therapy at the onset of stroke.

Cilostazol is an antiplatelet drug used for secondary prevention of cerebral infarction, and its administration does not increase the risk of bleeding or extend bleeding time. Moreover, in clinical settings, cilostazol causes significantly fewer hemorrhagic accidents than those caused by aspirin. We and others have reported that cilostazol has protective effects on vascular endothelial cells and that it suppresses the exacerbation of HT by tissue-type plasminogen activator.

In the present study, we obtained the following 4 results: (1) cilostazol (3 mg/kg, IP) prevented the exacerbation of HT caused by warfarin without affecting infarct volume; (2) survival rate was significantly higher in the combination therapy group than in the warfarin monotherapy group; (3) the mechanism whereby cilostazol prevented the exacerbation of HT might involve maintenance of the expression of tight junction proteins (claudin-5 and ZO-1) and VE-cadherin; and (4) combination therapy did not extend bleeding time.

We used the MCAO model to evaluate cerebral infarction because the resulting infarct volume was relatively stable and it provided a relevant model of cardioembolic ischemic stroke, where large blood vessels, including the middle cerebral artery, are occluded.
artery, are occluded. We determined the optimal timing and route of cilostazol administration in a previous study using a tissue-type plasminogen activator–induced HT model.12 We have also reported that a high dose of cilostazol (10 mg/kg, IP) suppressed infarct volume,10 but a lower dose (3 mg/kg, IP) did not,29 and we found similar results in the present study. However, swelling was exacerbated by warfarin administration, and this effect was suppressed by cilostazol (3 mg/kg, IP), resulting in the same severity of swelling as that seen in the vehicle group. These findings were in agreement with the results obtained for hemorrhage volume, which was about 10 times larger in the warfarin monotherapy group than in the vehicle group. Cilostazol (3 mg/kg, IP) reduced the hemorrhage volume to about one third. It also improved the survival rate of mice with warfarin-induced HT, which correlated with the reduction of HT volume.

However, a preliminary study (data not shown) indicated that HT was exacerbated by high-dose cilostazol administration (10 mg/kg, IP). High levels of cilostazol may enhance its antiplatelet effects, overriding its protective effects on vascular endothelial cells.

The neurological deficit scores up to 7 days after MCAO did not differ significantly among the groups (data not shown). This may reflect the lack of drug-related effects on infarct volume observed in the present study. This is in agreement with our previous study, where we found that the neurological deficit scores of mice with tissue-type plasminogen activator–induced HT did not worsen and were not altered by cilostazol (3 mg/kg, IP).12,29 Long-term studies may show greater intergroup differences in neurological deficit scores.

Our previous study indicated that cilostazol prevented tissue-type plasminogen activator–induced HT by suppression of MMP-9 expression.12 This finding suggested that MMP-9 was involved in BBB collapse, and that overexpression of MMP-2 or MMP-9 may exacerbate swelling and hemorrhage after brain injury, through the breakdown of collagen type IV in the vessel basement membrane.30–32 We therefore investigated MMP-2 and MMP-9 expression levels; however, no drug-related changes in MMP-2 or MMP-9 protein levels were found (Figure 1 in the online-only Data Supplement).

We quantified tight junction proteins and VE-cadherin expression levels because dissociation of these membrane proteins, which connect vascular endothelial cells, is known to be a direct cause of HT. In our previous studies, cilostazol was reported to increase claudin-5 and VE-cadherin expression levels, thereby protecting vascular endothelial cells.12,13 In the present study, ischemia and reperfusion were associated with reduced claudin-5, ZO-1, and VE-cadherin expression. This effect was prevented by treatment with cilostazol. We did not

Table 1. Tail Vein Bleeding Time in Mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bleeding Time, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>251.3±64.0</td>
</tr>
<tr>
<td>Warfarin</td>
<td>596.3±149.9</td>
</tr>
<tr>
<td>Warfarin+cilostazol (3 mg/kg)</td>
<td>600.0±135.1</td>
</tr>
</tbody>
</table>

This table shows the effects of warfarin and cilostazol on bleeding times. The bleeding time was significantly prolonged by warfarin administration but not by the combination of cilostazol (3 mg/kg, IP) and warfarin. Data are expressed as mean±SD (n=8 animals).
identify any warfarin-related changes in expression of these proteins, and the expression of occludin was not significantly changed in any group. These findings suggested that cilostazol reduced hemorrhage volume via a protective effect on vascular endothelial cells.

We also measured the plasma concentrations of cilostazol in the treated mice (Table 2) and found that the patterns in mice were similar to those reported previously in humans. Cilostazol is known to inhibit platelet aggregation without prolonging bleeding time, and the combination therapy did not prolong the prothrombin time or activated partial thromboplastin time as compared with warfarin alone. We also directly measured bleeding time by using the classic tail bleeding method, to confirm that the bleeding time was not extended by administration of warfarin and cilostazol, which would be a necessary factor for clinical use of this combination therapy. In agreement with previous results, we verified that the combination therapy did not prolong the bleeding time, and therefore, combined administration of warfarin and cilostazol (3 mg/kg, IP) may be clinically feasible.

These findings indicated that administration of cilostazol in the acute phase after ischemia might reduce HT, particularly in patients receiving anticoagulant therapy. Accordingly, the present experimental data may have important clinical implications.

Conclusions
The findings of this study indicated that the administration of cilostazol could reduce warfarin-induced HT in mice. This beneficial effect of cilostazol was associated with improved survival and effects on proteins important for BBB integrity, without any extension of bleeding time. These findings have important clinical implications, suggesting that if similar effects are found in humans, administration of cilostazol in the acute phase after ischemia may reduce HT in all patients, and would be particularly beneficial in those receiving anticoagulant therapy. Appropriate human clinical studies are necessary to confirm the efficacy and safety of this therapy.

Sources of Funding
This study received financial support from Otsuka Pharmaceutical Co Ltd to perform a collaborative research.

Disclosures
Dr Ito is an employee of Otsuka Pharmaceutical Co Ltd. The present study was evaluated by using cilostazol from his company.

Table 2. Plasma Cilostazol Concentrations

<table>
<thead>
<tr>
<th>Time After Cilostazol Treatment, h</th>
<th>Plasma Concentrations, ng/mL</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>2</td>
<td>6.3±5.5</td>
</tr>
<tr>
<td>4</td>
<td>1.9±3.2</td>
</tr>
<tr>
<td>8</td>
<td>6.3±5.5</td>
</tr>
</tbody>
</table>

This table shows plasma cilostazol concentrations of C57BL/6 mice. Data are expressed as mean±SD (n=3 per time point and experimental group).

References


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Supplemental Methods

1.1 Prothrombin Time-International Normalized Ratio Measurements

The PT-INR is a parameter for blood clotting, and the recommended PT-INR range in patients with atrial fibrillation during warfarin control is 2 to 3.\textsuperscript{1,2} In the present study, in order to reveal a more protective effect of cilostazol, the target PT-INR value was set to a value over the therapeutic range (ie, 2 or more).

After administering warfarin to the mice during each treatment period, the mice were anesthetized with pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan), and an abdominal incision was made. Blood samples (0.9 mL) were collected from the inferior vena cava by using a 1-mL syringe and a 25-gauge needle and gently transferred to 1.5-mL tubes containing 0.1 mL of 3.2% citrate. The tubes were then centrifuged for 5 min at 3,000 rpm, and the supernatants were transferred to new 1.5-mL tubes. Measurements of the PT-INR values (0 h, n = 6; 6 h, n = 7; 12 h, n = 8; 18 h, n = 10; 24 h, n = 10) were performed by Falco Biosystems Ltd. (Kyoto, Japan).

1.2 Measurement of Infarct Volume

At 21 h after reperfusion, the mice were decapitated. The brains were removed immediately and were cut to obtain slices with a thickness of 2 mm by using mouse brain matrix (RBM-2000C, Activational Systems, Warren, MI, USA). These slices were immersed in 2,3,5-triphenyl-tetrazolium chloride (2%; Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Infarct volume was measured (vehicle, n = 15; warfarin, n = 15; warfarin plus cilostazol 1 mg/kg, n = 7; warfarin plus cilostazol 3 mg/kg, n = 13) by an examiner blinded to treatment allocation by using Image J, as described previously.\textsuperscript{3}

1.3 Spectrophotometric Assay of Hemoglobin

At 21 h after reperfusion, the mice were injected ip with an overdose of pentobarbital sodium and transcardially perfused with cold saline. The brains were removed immediately, and a 2- to 10-mm portion of the forebrain was excised and sectioned into the normal side and the infarction side by using the mouse brain matrix. HT was quantified (sham, n = 6; vehicle, n = 9; warfarin, n = 14; warfarin plus cilostazol 1 mg/kg, n = 9; warfarin plus cilostazol 3 mg/kg, n = 15) by spectrophotometric assay of the hemoglobin contained in the brains.\textsuperscript{4} After addition of saline to each sample (10 mL/g), the brain tissues were homogenized and centrifuged for 30 min at 13,000 g. A 50-μL aliquot of the supernatant was then mixed with 200 μL of reagent (QuantiChrom Hemoglobin Assay Kit; BioAssay Systems, CA, USA). After 15 min, optical density was read at 400 nm by using a spectrophotometer (Skan It RE for Varioskan 133 Flash
2.4; Thermo Fisher Scientific, Waltham, MA, USA). Unstained cryosections were prepared as reported previously.5

1.4 Evaluation of Survival Rate
We confirmed the long-term effects of each therapy by evaluating the survival rate for 7 days after focal cerebral ischemia (warfarin, n = 12; warfarin plus cilostazol 3 mg/kg, n = 10). We measured the body weight of each mouse as an indicator of general health during the experiment and monitored the survival rate for 7 days. We considered the mice to be dead when the righting reflex was no longer observed.

1.5 Evaluation of Matrix Metalloproteinase-2 and 9 Expression
Mice were decapitated, and the brains were removed immediately 21 h after reperfusion. A 2- to 10-mm portion was cut from the forebrain by using brain matrix without perfusion. Sham-operated mice were used as the control group. Tissues of the infarction side were homogenized in 10 mL/g tissue ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, and a protease/phosphatase inhibitor mixture). A 5-μg protein sample was subjected to electrophoresis on a 5% to 20% gradient SDS-polyacrylamide gel (SuperSep Ace; Wako Pure Chemicals, Osaka, Japan), and the separated proteins were subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA, USA). For western blotting, the following primary antibodies were used: polyclonal antibody to MMP-2 (1/2000), MMP-9 (1/2000; Millipore Corporation), and monoclonal antibody to β-actin (1:10000; Sigma-Aldrich). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Lumino Imaging Analyzer (FAS-1000; Toyobo Engineering, Osaka, Japan) and Gel Pro Analyzer (Media-Cybernetics, Inc., Bethesda, MD, USA) were used to measure the band intensities. (sham, n = 6; vehicle, n = 14; warfarin, n = 11; warfarin plus cilostazol 3 mg/kg, n = 14).

1.6 Evaluation of Tight Junction Proteins and VE-cadherin Expression
The mice were decapitated, and the brains were removed immediately 6 h after reperfusion because we found that the expression of these proteins tended to be reduced at 6 h after reperfusion in our preliminary study (data not shown). For western blotting, the following primary antibodies were used: polyclonal antibody to ZO-1 (1:250), occludin (1:2000), claudin-5 (1:200; Santa Cruz, CA, USA), VE-cadherin (1:2000; Abcam, Cambridge, UK), and monoclonal antibody to β-actin (1:10000) (sham, n = 9;
vehicle, n = 9; warfarin, n = 9; warfarin plus cilostazol 3 mg/kg, n = 8).

1.7 Measurement of Bleeding Time

At 18 h after warfarin administration, the mice were anesthetized with 1.5% isoflurane (in 80% N₂O/20% O₂) by using an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Cilostazol was administered 2 h before anesthesia. Body temperature was maintained at 37 to 37.5°C with the aid of a heating pad and heating lamp. A transverse incision was made with a scalpel over the lateral tail vein at a position where the diameter of the tail was 2.5 mm. The flow of blood was blotted with filter paper every 30 s, and the time was measured until blood no longer stained the filter paper (normal, n = 8; warfarin, n = 8; warfarin plus cilostazol 3 mg/kg, n = 8).

1.8 Statistical Analysis

We used Statview version 5.0 (SAS Institute Inc., Cary, NC, USA) for statistical analysis. Data are presented as means ± SD (standard deviation) or SEM (standard error of the mean). Statistical comparisons were made using Student’s t-test, Tukey’s t-test, or Log-rank t-test. P < 0.05 was considered to indicate statistical significance.
Supplementary Figure I. Western blot analysis of matrix metalloproteinase-2 and 9 at 21 h after reperfusion.

The effects of warfarin and cilostazol on expression of matrix metalloproteinase-2 and 9. Data are expressed as mean ± SEM (**P < 0.01 vs Sham; Student’s t-test, n = 6 to 14 animals); CSZ, cilostazol
Supplemental References


