Fasudil and ozagrel in combination show neuroprotective effects on cerebral infarction after murine middle cerebral artery occlusion

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a) **Running title:** Fasudil and Ozagrel in combination show neuroprotection.

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c) **Number of text pages:** 35

   Number of tables: 1

   Number of figures: 9

   Number of references: 28

   Number of words in Abstract: 241

   Number of words in Introduction: 485

   Number of words in Discussion: 731

d) **Abbreviation:** AUC, area under the plasma concentration-time curve; CBF, cerebral blood flow; Cmax, maximum concentration; e-NOS, endothelial nitric oxide synthase; MLC, myosin light chain; MCAO, middle cerebral artery occlusion; PGI2, prostacyclin; PVDF, polyvinylidene difluoride; P-eNOS, phosphorylated-eNOS; ROCK, Rho kinase; SAH, subarachnoid hemorrhage; T1/2, elimination half-life; Tmax, time to the maximum concentration; TTC, triphenyltetrazolium chloride; TXA2, Thromboxane A2.

e) **Recommended section:** Neuropharmacology
ABSTRACT

Rho kinase (ROCK), one of the serine/threonine kinases, is involved in pathologic conditions and its activation causes neuronal cell death. Fasudil, a selective ROCK inhibitor, has been reported to cause increased cerebral blood flow (CBF) in the ischemic brain and to protect against neuronal cell death by inhibiting ROCK. Ozagrel, a thromboxane A₂ synthase inhibitor, inhibits platelet aggregation and causes vasodilatation, thereby increasing CBF in cerebral thrombosis. The present study evaluates the combination therapy of fasudil and ozagrel on focal brain ischemia induced by middle cerebral artery occlusion (MCAO) in mice. Each monotherapy of fasudil at 10 mg/kg, i.p. and ozagrel at 30 mg/kg, i.p. significantly reduced cerebral infarction. The combination therapy of fasudil (3 mg/kg, i.p.) and ozagrel (10 mg/kg, i.p.), which are non-effective doses respectively, resulted in reduction of cerebral infarction and the protective effect was observed up to 5 min, but not 3 h, after reperfusion. Regional CBF after MCAO and phosphorylation of endothelial nitric oxide synthase (e-NOS) significantly increased in response to the combination therapy, whereas these effects were not observed with monotherapy of either drug. The protective effect of combination treatment was antagonized by the treatment of a NOS inhibitor, nitro-L-arginine methyl ester hydrochloride (L-NAME). These findings
indicate that the combination treatment of fasudil and ozagrel exhibits additive effects for neuroprotection after MCAO. These findings indicate that the combination treatment of fasudil and ozagrel may be useful as a potential therapeutic strategy for treatment of stroke.
Introduction

Cerebral infarction is a life-threatening disease. Many patients who experience cerebral infarction suffer seriously from its aftereffects. Therefore, understanding its pathology and finding effective treatments have become very important. Currently, treatment of cerebral infarction is primarily aimed at improvement of cerebral blood flow (CBF). For example, tissue-plasminogen activator (t-PA) is a most effective therapeutic agent for acute cerebral infarction. However, t-PA can sometimes cause fatal hemorrhagic complications (Wahlgren et al., 2007). Therefore, safer therapeutic agents for cerebral infarction are still needed.

Rho kinases (ROCKs) are serine/threonine kinases that exist as two isoforms, ROCK I and ROCK II (Riento et al., 2003). ROCKs are activated by binding to the active GTP bound form of Rho (Amano et al., 2000). ROCKs are the most important elements involved in endothelial nitric oxide synthase (eNOS) activity, cell migration, and hyperviscosity (Shibuya et al., 2005). Previous studies suggest that ROCKs play an important role in pathologic conditions such as coronary spasm (Masumoto et al., 2002), hypertension (Masumoto et al., 2001), vascular inflammation and remodeling (Kataoka et al., 2002), and arteriosclerosis (Miyata et al., 2000).

Fasudil, a Rho kinase inhibitor, is already in use in several countries as a treatment
for cerebral vasospasms occurring after subarachnoid hemorrhage (SAH) (Sayama et al., 2006). In a previous report, fasudil showed neuroprotective effects following cerebral ischemia in animal models (Rikitake et al., 2005). In humans, treatment with fasudil within 48 h of onset of acute ischemic stroke significantly improved the patients’ clinical outcome (Shibuya et al., 2005). These findings indicate that fasudil is one of the most promising agents for treatment of cerebral infarction.

Ozagrel, a thromboxane A2 synthetase inhibitor, is presently used in several countries for the treatment of acute cerebral infarction or to prevent cerebral vasospasms after SAH. Thromboxane A2 (TXA2) promotes platelet aggregation by increasing Ca$^{2+}$ influx and facilitating the release of ADP (Aiken, 1984, Bosia et al., 1988). Ozagrel decreases TXA2 production and has antiplatelet and antithrombotic effects (Terashita et al., 1995). Ozagrel also causes a secondary increase in prostacyclin (PGI2) production, followed by an inhibition of TXA2-synthetase (Komatsu et al., 1986). Increased PGI2 causes a vasodilatation effect and increases CBF (Pickard et al., 1980). Ozagrel has been reported to improve motor coordination in rats following experimental stroke (Ichikawa et al., 1999).

In the present study, we hypothesized that a combination therapy of fasudil and ozagrel would show a greater neuroprotective effect following cerebral ischemia than
would either drug administered as a monotherapy. The combined effect of fasudil and ozagrel has previously been evaluated in patients with delayed cerebral vasospasm after SAH (Suzuki et al., 2008). However, similar evaluation has not yet been documented with respect to cerebral infarction. Since the mechanism of action of ozagrel is different than that of fasudil, the purpose of the present study was to evaluate the neuroprotective effects of combined fasudil and ozagrel therapy against middle cerebral artery occlusion (MCAO)-induced ischemic damage in mice.

Material and methods

Animal preparation

The experimental designs and all procedures were in accordance both with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Animal Care Guidelines issued by the Animal Experimental Committee of Gifu Pharmaceutical University. All experiments were performed using male ddY mice (5-8 weeks old, Japan SLC Ltd., Shizuoka, Japan). Every effort was made to minimize the number of animals used and their suffering.

Drugs
Fasudil and ozagrel, kindly donated by Asahi Kasei Co. Ltd. (Tokyo, Japan), were dissolved in saline and administered intraperitoneally. Other drugs, including 2,3,5-triphenyltetrazolium chloride (TTC), nitro-L-arginine methyl ester hydrochloride (L-NAME), pentobarbital sodium, and isoflurane, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), Sigma-Aldrich Co., Nissan Kagaku (Tokyo, Japan), and Merck Hoei Ltd. (Osaka, Japan), respectively.

**Plasma concentration and pharmacokinetic parameters**

Fasudil (3 and 10 mg/kg) was injected intraperitoneally. Blood samples were collected from the retro-orbital venous plexus 5, 15, 30, 60, and 180 min after dosing. Concentrations of fasudil and hydroxyfasudil in plasma were measured by LC-MS/MS (API 4000, LC-MS/MS System, Applied Biosystems/MDR Sciex, Foster City, CA, USA). The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal rule with extrapolation. The maximum concentration (Cmax) and the time to the maximum concentration (Tmax) were derived directly from individual measurements taken. The elimination half-life (T1/2) was calculated by linear regression.
Drug treatments

Fasudil and ozagrel were diluted in saline. For the evaluation of dose-dependency, mice were injected with fasudil (1, 3, or 10 mg/kg, i.p.) and ozagrel (3, 10, or 30 mg/kg, i.p.) at 10 min following MCAO. For the evaluation of the combination therapy, mice were injected with submaximal (non-effective for ischemic infarction) doses of fasudil (3 mg/kg i.p.) and ozagrel (10 mg/kg i.p.) concomitantly at 10 min after MCAO. For the evaluation of therapeutic time window of combination therapy, mice were injected with submaximal doses of fasudil (3 mg/kg i.p.) and ozagrel (10 mg/kg i.p.) at 5 min or 3 h after 2 h ischemia-reperfusion. For the evaluation of L-NAME with combined ozagrel and fasudil therapy, L-NAME (3 mg/kg i.v.) was treated immediately after the induction of MCAO, and then fasudil (3 mg/kg i.p.) and ozagrel (10 mg/kg i.p.) were treated concomitantly at 10 min after MCAO.

Surgery

Mice were anesthetized with 2 to 3% isoflurane (for induction) and maintained with 1.0 to 1.5% isoflurane in 70% N₂O and 30% O₂ via a facemask (Soft Lander; Sin-ei Industry, Saitama, Japan). Focal cerebral ischemia was induced [using an 8-0 nylon monofilament (Ethicon, Somerville, NJ, USA) coated with silicone hardener mixture]
(Xantpren; Bayer Dental, Osaka, Japan)] via the internal carotid artery, as described by Hara et al. (1996). Briefly, a coated filament was introduced into the left internal carotid artery through the common carotid artery and then advanced up to the origin of the anterior cerebral artery via the internal carotid artery, so as to occlude the middle cerebral artery and posterior communicating artery. At the same time, the left common carotid artery was occluded. Anesthesia did not exceed 10 min. After 2 h of occlusion, the animal was reanesthetized briefly and reperfusion was initiated via withdrawal of the monofilament. After surgery, the mice were kept for another 24 h in a cage under a heat lamp, which maintained the cage temperature at between 29 and 30°C. Thereafter, the mice were kept in the preoperative condition (24 ± 2°C) until sampling.

**Physiological monitoring**

During surgery and ischemia, the body temperature of all animals was maintained between 37.0 and 37.5°C with the aid of a heat lamp and heating pad. In randomly selected animals, the left femoral artery was cannulated and blood pressure was measured during the preparation, with mean systemic arterial blood pressure (Power Laboratory; AD Instrument, Nagoya, Japan) being measured for 3-min periods starting 10 min before and ending 30 min after MCAO. Arterial blood samples taken 30 min
before and 30 min after the induction of ischemia were analyzed for pH and partial pressures of oxygen (PaO$_2$) and carbon dioxide (PaCO$_2$) (i-STAT 3G; Abbott Point-of-Care Inc., East Windsor, NL, USA). Regional cerebral blood flow (CBF) was determined by laser-Doppler flowmetry (Omegaflow flo-N1; Omegawave Inc., Tokyo, Japan) using a flexible 0.5-mm fiber-optic extension to the master probe. The tip of the probe was fixed to the intact skull over the ischemic cortex (2 mm posterior and 6 mm lateral to bregma).

**Analysis of cerebral infarction**

At 24 h after MCAO, mice were given an overdose of pentobarbital sodium, then decapitated. The forebrain was divided into five coronal 2-mm sections using a mouse brain matrix (RBM-2000C; Activational Systems, Warren, MI, USA). These slices were immersed for 20 min in a 2% solution of triphenyltetrazolium chloride (TTC) in normal saline at 37˚C, then fixed in 10% phosphate-buffered formalin at 4˚C. The TTC reacts with intact mitochondrial respiratory enzymes to generate a bright red color that contrasts with the pale color of the infarction. The caudal face of each slice was photographed. The area of the infarction (unstained) in the left cerebral hemisphere was traced and measured using Image J (http://rsb.info.nih.gov/ij/download.html), and the
infarction volume per brain (mm³) was calculated from the measured infarction area.

**Neurological deficits**

Mice were tested for neurological deficits at 24 h or 72 h after MCAO. These were scored as described in our previous study (Hara et al., 1996): 0, no observable neurological deficits (normal); 1, failure to extend the right forepaw (mild); 2, circling to the contralateral side (moderate); 3, loss of walking or righting reflex (severe); 4, dead.

**Western blot analysis**

Mice were deeply anesthetized and decapitated at 6 h after MCAO. The brain was quickly removed and an 8-mm coronal section cut from the left hemisphere (between 2 and 10 mm from the frontal end of the forebrain). Samples were homogenized in RIPA buffer (Sigma-Aldrich) with 1% Triton X-100 and a protease/phosphatase inhibitor mixture. Homogenates were centrifuged at 14,000 × g for 40 min at 4°C. A 5 µg aliquot of protein was subjected to 5-20 %-gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SuperSep Ace; Wako, Osaka, Japan) and separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P;
Millipore, Billerica, MA, USA). For immunoblotting, the following primary antibodies were used: polyclonal antibody to nitric oxide synthase (endothelial) (1:1000 dilution; Funakoshi, Tokyo, Japan), phosphor-endothelial nitric oxide synthase (eNOS) (Ser1177) antibody (1:1000 dilution; Cell Signaling, Danvers, MA, USA), monoclonal anti-β-actin (1:1000 dilution; Sigma-Aldrich). The secondary antibody was anti-rabbit HRP-conjugated IgG (1:2000 dilution) and anti-mouse HRP-conjugated IgG (1:2000 dilution). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA). The band intensity was measured using a Lumino imaging analyzer (LAS-4000; Fuji Film, Tokyo, Japan).

Cell culture

Human aortic endothelial cells (Lonza Walkersville Inc., Walkersville, MA, USA) were cultured in 12-well plates in Human Endothelial SFM basal medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum and growth factors (EGM-2: Lonza Walkersville Inc., Walkersville, MA, USA). The medium was changed to Human Endothelial SFM basal medium with 0.5% fetal bovine serum and incubated with hydroxyfasudil and/or ozagrel for 1 h.
Measurement of phosphorylation of myosin light chain

Human aortic endothelial cells were lysed with SDS/Urea lysis buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated with anti-myosin light chain (MLC) antibody (Santa Cruz, Santa Cruz, CA, USA), anti-phospho-MLC antibody (Cell Signaling, Danvers, MA, USA) or anti-actin antibody (Sigma-Aldrich). The Rho-kinase activity was expressed as the ratio of the staining density of anti-phospho-MLC antibody to that of anti-MLC antibody.

Statistical analysis

Data are presented as the means ± S.E.M. Statistical comparisons were made using a one-way ANOVA followed by a Student’s t-test or Dunnett’s test using Statview version 5.0 (SAS Institute Inc., Cary, NC, USA), with P<0.05 being considered as statistically significant.

Results

Plasma concentrations and pharmacokinetic parameters

The plasma concentration-time curves and the pharmacokinetic parameters for
fasudil and hydroxyfasudil are shown in fig. 1 and table 1. Fasudil, the parent drug, and hydroxyfasudil, its metabolite, were found following intraperitoneal administrations of fasudil (3 or 10 mg/kg). Plasma concentrations of fasudil at 60 and 180 min following a 3 mg/kg administration, and at 180 min following a 10 mg/kg administration, were below detectable limits. The Cmax values of hydroxyfasudil were approximately 100% and 134% of those of fasudil when fasudil was administered at 3 and 10 mg/kg, respectively. The AUC value of hydroxyfasudil was approximately 4 times higher than that of fasudil. As can be understood from Tmax and T1/2, hydroxyfasudil was detectable in plasma for a longer period than was fasudil. The T1/2 value of hydroxyfasudil was approximately 4 times longer than that of fasudil.

Effects of a single administration of fasudil on ischemic damage

We first evaluated the dose-dependent effects of a single treatment of fasudil on ischemic damage. At 24 h after MCAO, an ischemic zone was consistently identified in the left cerebral hemisphere. No mice had died at 24 h after MCAO. The TTC staining results showed no clear differences between the fasudil (1 or 3 mg/kg, i.p.) and vehicle treatment groups (Fig. 2A to C). However, fasudil at 10 mg/kg, i.p., administered at 10 min after MCAO, decreased the cerebral infarction at 24 h after MCAO (Fig. 2D).
Fasudil at 10 mg/kg, i.p., but not at 1 or 3 mg/kg, improved the neurological deficits (Fig. 2E). Based on infarction measurements, fasudil significantly reduced both the infarct area and volume in a dose-dependent manner, with significant effects seen at 10 mg/kg (Fig. 2F and G).

Effects of a single administration of ozagrel on ischemic damage

We next evaluated the effects of a single treatment of ozagrel on ischemic damage. No mice had died at 24 h after MCAO. The TTC staining results showed no clear difference between the ozagrel (3 or 10 mg/kg, i.p., administered at 10 min after MCAO) and vehicle treatment groups (Fig. 3A to C). However, ozagrel at 30 mg/kg, i.p., decreased the cerebral infarction at 24 h after MCAO (Fig. 3D). Ozagrel at 30 mg/kg, i.p., but not at 3 or 10 mg/kg, improved the neurological deficits (Fig. 3E). Based on infarction measurements, ozagrel reduced both the infarct area and volume in a dose-dependent manner (Fig. 3F and G). Ozagrel at 30 mg/kg, i.p. was particularly effective at significantly reducing both the infarct area and volume.

Effects of combined fasudil and ozagrel therapy on ischemic damage

Next, we evaluated the effects of the combined fasudil and ozagrel therapy. Neither
ozagrel at 10 mg/kg nor fasudil at 3 mg/kg caused any significantly decrease the infarct area and volume when supplied singly (Figs. 2 and 3). Thus, we administered ozagrel and fasudil together using each of these non-effective doses. No mice had died at 24 h after MCAO. The TTC staining results showed no clear difference between vehicle-, fasudil-, or ozagrel-treated groups (Fig. 4A to C). On the other hand, the combination therapy decreased the cerebral infarction (Fig. 4D) and improved the neurological deficits (Fig. 4E). Based on infarction measurements, the combination therapy significantly reduced both the infarct area and volume (Fig. 4F and G). Moreover, significant effects of the combination therapy on infarct area and volume could be seen both in the cortex and in the subcortex (striatum) (Fig. 4H and I).

**Effect of combined ozagrel and fasudil on a therapeutic time window**

We examined the therapeutic time window of combined ozagrel and fasudil therapy. When administered at 5 min after reperfusion, combined ozagrel and fasudil therapy significantly reduced the infarct area and volume at 24 h after MCAO (22 h reperfusion), but not at 3 h after reperfusion (Fig 5A and B).

**Physiological parameters**
No significant differences were detected in mean arterial blood pressure, heart rate, PaCO₂, or PaO₂ among all of the groups (data not shown). We measured rCBF of mice by laser-Doppler flowmetry during MCAO and found that rCBF of all mice significantly decreased to about 30–40% of the previous value (Fig. 6). After reperfusion, rCBF of the combination group significantly increased compared with the value observed during MCAO (Fig. 6D), but this response was not seen in any of the other groups (Fig. 6A to C).

**Western blotting of eNOS**

Other studies have reported that eNOS is phosphorylated and activated in the ischemic brain hemisphere and that phosphorylated-eNOS (P-eNOS) exerts neuroprotective effects (Osuka et al., 2004). We measured eNOS and P-eNOS in penumbra lesions of the ischemic brain hemisphere. P-eNOS was more prominent in the combination therapy group than in the other groups and there was no difference among the other groups (Fig. 7A). In a quantitative analysis, whereas fasudil or ozagrel did not increase P-eNOS, P-eNOS was significantly greater in the combination therapy group than in the control group (Fig. 7B). We also evaluated the ratio of P-eNOS/eNOS protein. In the result, control: 1.000±0.093, vehicle: 1.119±0.254, fasudil: 1.088±
0.184, ozagrel: 1.232±0.227, combination: 1.506±0.324 (each n=5). It tended to increase the ratio, but did not reach the significant level (control vs. combination; p=0.193, Student’s t-test).

**Antagonistic effect of L-NAME with combined ozagrel and fasudil on ischemic damage**

As P-eNOS was increased in combined ozagrel and fasudil therapy group (Fig. 7), we evaluated effect of a NOS inhibitor, L-NAME, with combined ozagrel and fasudil treatment against MCAO-induced ischemic damage. There was no significant difference between L-NAME with combined ozagrel and fasudil group and vehicle group in infarct area or volume at 24 h after MCAO (Fig. 8A and B).

**Effect of hydroxyfasudil and ozagrel on myosin light chain phosphorylation in human aortic endothelial cells**

Hydroxyfasudil at 30 μM inhibited phosphorylation of MLC, whereas at 1 mM ozagrel had no effect on MLC phosphorylation, nor did it alter the inhibitory effect of hydroxyfasudil (Fig.9).
Discussion

In the present study, we investigated the effects of fasudil and ozagrel, administered singly or in combination, in a murine model of transient MCAO. Fasudil and ozagrel both exhibited dose-dependent neuroprotective effects, with doses of 10 mg/kg, i.p., for fasudil and 30 mg/kg, i.p. for ozagrel significantly reducing both the infarct volume and the neurological deficits following MCAO. Neither fasudil at 3 mg/kg nor ozagrel at 10 mg/kg showed any neuroprotective effects, but when combined as a therapy at these doses, cerebral infarction was reduced. The combination therapy was able to prevent neuronal cell death even when it was injected at up to 5 min, but not at 2 h, after ischemia/reperfusion. The combination therapy also increased CBF after reperfusion and activated the phosphorylation of eNOS. Furthermore, the neuroprotective effect of the combination therapy was inhibited by an eNOS inhibitor, L-NAME.

Shimokawa (2002) has reported that abnormal ROCK function may contribute to the pathogenesis of ischemic stroke. ROCK activity has also been reported to increase in the ischemic brain hemisphere (Feske et al., 2009) and, in our previous study, fasudil inhibited ROCK and reduced ischemic damage following MCAO (Yamashita et al., 2007). eNOS is activated by the phospahtidylinositol-3 kinase (PI-3K)/Akt pathway.
Ming et al. reported that Rho/ROCK pathway negatively regulated eNOS phosphorylation through inhibition of PI-3K/Akt (Ming et al., 2002). Rikitake et al. have reported that fasudil increased CBF by upregulation of eNOS and decreased cerebral infarction in mice (Rikitake et al., 2005). On the other hand, ozagrel has been reported to decrease platelet aggregation and increase CBF by decreasing levels of TXA2 (Oishi et al., 1996). Thus, while fasudil appears to act primarily on vascular smooth muscle cells, ozagrel mainly acts on platelets.

In the present study, each low doses of fasudil (3 mg/kg) and ozagrel (10 mg/kg) had no significant neuroprotective effects, whereas combined therapy at these doses decreased cerebral infarction, significantly increased CBF after reperfusion, and increased p-eNOS levels in the ischemic cerebral hemisphere. Xavier et al. reported that inhibition of TXA2 and PGI2 synthesis increased P-eNOS protein, but not eNOS release in mesenteric artery (Xavier et al. 2010). The detailed mechanism is still unclear, however ozagrel may activate phosphorylation of eNOS by inhibiting of TXA2 synthase. Furthermore, PGI2 is known to collaborate with the system consisting of eNOS/NO (Gryglewski, 2008). Ozagrel may indirectly activate phosphorylation of eNOS. P-eNOS is related to the production of NO, while NO levels correlate with the extent of neuroprotection or vasodilatation. These findings indicate that the effect of ozagrel may
be to modify the effect of fasudil on eNOS-activation. However, further experiments will be needed to clarify the detailed mechanism.

Hydroxyfasudil, an active metabolite of fasudil, was found following administration of fasudil (10 mg/kg, i.p.). The Cmax and AUC values of hydroxyfasudil were approximately 134% and 396% of the parent drug, respectively. Hydroxyfasudil and fasudil strongly inhibit Rho-kinase with almost equally potency (Yano, et al., 2008) and both can improve cerebral infarct in mice (Shin, et al., 2007; Yamashita, et al., 2007). Thus, hydroxyfasudil contributes to the potency of fasudil.

Following an administration of fasudil (10 mg/kg, i.p.), the AUC value of fasudil plus hydroxyfasudil was 1907.1 ng·hr/ml in mice. Following intravenous infusion of fasudil (60 mg/60 min; approximately 1 mg/kg), the AUC value of fasudil plus hydroxyfasudil was 1319.8 ng·hr/ml in elderly healthy volunteers (Shibuya et al., 2005). The AUC value of fasudil (1 mg/kg, a dose that showed clinical effectiveness in patients with acute stroke) in humans was similar to that after an i.p. administration of fasudil (10 mg/kg, i.p.) in mice.

Rho-kinase increases the phosphorylation of MLC through inhibition of MLC phosphatase activity by phosphorylation of its myosin binding subunit (Sato et al., 2000). In the present study, hydroxyfasudil inhibited the phosphorylation of MLC in
endothelial cells, while ozagrel showed no inhibition of MLC phosphorylation and did
not alter the inhibitory effect of hydroxyfasudil. Ozagrel did not affect ROCK activity
and did not alter the effect of fasudil. Suzuki et al. (2008) demonstrated that ozagrel did
not inhibit ROCK activity in an enzyme immunoassay system. It is interesting that
co-administration of fasudil and ozagrel increased phosphorylation of eNOS above that
seen with single administrations in the mouse ischemia model. This suggests an as yet
unknown in vivo mechanism by which ozagrel can enhance the phosphorylation of
eNOS, although ozagrel does not have the potential to directly inhibit ROCK activity.

**Conclusion**

These findings indicate that a combination therapy of fasudil and ozagrel exhibits a
greater neuroprotective effect on cerebral infarction after murine MCAO than does
either drug administered as a monotherapy. The neuroprotective effect of combined
therapy expressed at least 5 min after ischemia/reperfusion. The effect is apparently
achieved by increasing eNOS and consequently CBF. Hence, a combination therapy of
fasudil and ozagrel may have potential as a novel therapy for treatment of stroke in
humans.
Acknowledgement

We thank Shino Bito, Masashi Ishiguro, and Yuji Ohtsuka (Asahi Kasei Pharma Co.Ltd.) for their expert technical assistance with PK analysis and cell assays.

Authorship contributions

HH participated in research design. AK, JH, and KK conducted experiments. IH and TI contributed to new reagents or analytic tools. KT and MS performed data analysis. AK, JH, and HH wrote or contributed to the writing of the manuscript.
References


Figure legends

Figure 1. Plasma concentration-time curves for fasudil, the parent drug, and hydroxyfasudil, its metabolite.

(A) Plasma concentration of fasudil and hydroxyfasudil following administration of fasudil at 3 mg/kg, i.p.; (B) Plasma concentrations of fasudil and hydroxyfasudil after administration of fasudil at 10 mg/kg, i.p. Plasma concentrations of fasudil were below detectable limits at 60 and 180 min following administration at 3 mg/kg, i.p., and at 180 min following administration at 10 mg/kg, i.p.

Figure 2. Effects of fasudil on infarction 24 h after middle cerebral artery occlusion (MCAO) (at 22 h after reperfusion) in mice.

(A) to (D) TTC staining of coronal brain sections (4 mm from olfactory bulb) [(A) Vehicle; (B) Fasudil 1 mg/kg, i.p.; (C) Fasudil 3 mg/kg, i.p.; (D) Fasudil 10 mg/kg, i.p.]. Fasudil dose-dependently decreased the infarct area (white area). (E) Effects of fasudil on neurological deficits (assessed at 24 h after MCAO). Fasudil 10 mg/kg, i.p. significantly improved the neurological score. Horizontal bars indicate the means. *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9). (F and G) Effects of fasudil on brain infarct area (F) and volume (G) measured at 24 h after MCAO (at 22 h after reperfusion).
reperfusion). Fasudil dose-dependently decreased the infarct area and volume and significantly decreased these at 10 mg/kg, i.p. *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9).

Figure 3. Effects of ozagrel on infarction at 24 h after middle cerebral artery occlusion (MCAO) (at 22 h after reperfusion) in mice.

(A) to (D) TTC staining of coronal brain sections (4 mm from olfactory bulb) [(A) Vehicle; (B) Ozagrel 3 mg/kg, i.p.; (C) Ozagrel 10 mg/kg, i.p.; (D) Ozagrel 30 mg/kg, i.p.]. Ozagrel dose-dependently decreased the infarct area (white area). (E) Effects of fasudil on neurological deficits (assessed at 24 h after MCAO). Ozagrel 30 mg/kg, i.p., significantly improved the neurological score. Horizontal bars indicate the means. *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9). (F and G) Effects of ozagrel on the brain infarct area (F) and volume (G) measured at 24 h after MCAO (at 22 h after reperfusion). Ozagrel dose-dependently decreased infarct area and volume and significantly decreased these at 30 mg/kg, i.p. *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9).

Figure 4. Effects of combined ozagrel and fasudil therapy on infarction at 24 h after
middle cerebral artery occlusion (MCAO) (at 22 h after reperfusion) in mice.

(A) to (D) TTC staining of coronal brain sections (4 mm from olfactory bulb). [(A) Vehicle; (B) Fasudil 3 mg/kg, i.p.; (C) Ozagrel 10 mg/kg, i.p.; and (D) Fasudil 3 mg/kg and ozagrel 10 mg/kg, i.p.]. (E) Effects of fasudil, ozagrel, and the combination therapy of fasudil and ozagrel on neurological deficits (assessed at 24 h after MCAO). The combination therapy significantly improved the neurological score. Horizontal bars indicate the means. *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9). (F and G) Effects of fasudil on the brain infarct area (F) and volume (G) measured at 24 h after MCAO (22 h after reperfusion). Combination therapy of fasudil and ozagrel significantly decreased the infarct area and volume in a dose-dependent manner. *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9). (H) Brain infarct area in cortex and subcortex (striatum) at 24 h after MCAO (at 22 h after reperfusion). Combination therapy significantly decreased infarct area in both the cortex and subcortex (striatum). *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9). (I) Brain infarct volume in cortex and subcortex at 24 h after MCAO (22 h after reperfusion). Combination therapy significantly decreased the infarct volume in both the cortex and subcortex. *P<0.05 vs. vehicle (Dunnett’s test) (n=8 or 9).

Figure 5. Effect of combined ozagrel and fasudil therapy after middle cerebral artery
occlusion (MCAO) on a therapeutic time window.

Combination therapy was conducted after 2 h ischemia-reperfusion (I/R). Effect of combination treatment on infarct area (A) and infarct Volume (B) measured at 24 h after middle cerebral artery occlusion (MCAO) (at 22 h after reperfusion). **P<0.01 vs. vehicle (Dunnett’s test) (n=7 or 8).

Figure 6. Changes in regional cerebral blood flow (rCBF) during middle cerebral artery occlusion (MCAO) and reperfusion.

The rCBF value (% of before) was measured by laser-Doppler flowmetry. (A) Vehicle; (B) Fasudil 3 mg/kg, i.p.; (C) Ozagrel 10 mg/kg, i.p.; (D) Combination treatment: Fasudil 3 mg/kg, i.p. and ozagrel 10 mg/kg, i.p. The rCBF significantly decreased following MCAO in all groups. The rCBF value significantly increased after reperfusion in the combination group, but not significantly in any other groups. **P<0.01 vs. before ischemia, *P<0.05 vs. during ischemia (Student’s t-test) (n=4)

Figure 7. Western blot analysis of P-eNOS.

(A) P-eNOS tended to increase after middle cerebral artery occlusion (MCAO), but not significantly. It was more prominent in all therapeutic groups than it was in the control.
No significant difference was seen for eNOS in any group. (B) Quantitative analysis of Western blotting of P-eNOS: P-eNOS in the combined treatment group was greater than in the control group. * P<0.05 vs. Control (Student’s t-test) (n=6).

Figure 8. Antagonistic effect of L-NAME with combined ozagrel and fasudil therapy on infarction after middle cerebral artery occlusion (MCAO) (at 22 h after reperfusion).

Effect of L-NAME treated with combined fasudil and ozagrel on infarct area (A) and volume (B). There was no significant reduction in infarct area or volume compared with vehicle treated group. (n=7 or 8).

Figure 9. Western blotting of myosin light chain (MLC) and phosphorylated MLC.

Hydroxyfasudil (HF) inhibited the phosphorylation of MLC in endothelial cells. Ozagrel did not inhibit the phosphorylation of MLC, and did not alter the inhibitory effect of hydroxyfasudil.
Table 1 Pharmacokinetic parameters for fasudil and hydroxyfasudil in mice after an intraperitoneal administration of fasudil.

<table>
<thead>
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<th>Parameters</th>
<th>Fasudil</th>
<th>Hydroxyfasudil</th>
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<tr>
<td></td>
<td>3 mg/kg</td>
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<tr>
<td></td>
<td>3 mg/kg</td>
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<td>AUC (ng·hr/ml)</td>
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<td>T1/2 (min)</td>
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<tr>
<td></td>
<td>51.4</td>
<td>36.4</td>
</tr>
</tbody>
</table>

Values are represent mean (n=4).
Fig. 1

A

- Fasudil 3mg/kg i.p.
- Hydroxyfasudil

B

- Fasudil 10mg/kg i.p.
- Hydroxyfasudil

Plasma concentration (ng/ml)

Time after administration
Fig. 2

A B C D E

F G

Vehicle 1 3 10 mg/kg
Fasudil

Neurological score

Coronal slices

Infarct area (mm²)

Infarct volume (mm³)

Vehicle 1 3 10 mg/kg
Fasudil

*
Fig. 3

A, B, C, D: Images of brain slices.

E: Graph showing neurological score.

F: Graph showing infarct area (mm²) across coronal slices.

G: Graph showing infarct volume (mm³) for different doses of Ozagrel.
Fig. 4

A-C: Representative images of brain sections showing differences in tissue appearance.

D: Comparison of neurological scores across treatment groups.

E: Graph showing vehicle, fasudil, ozagrel, and combination treatment effects.

F: Infarct area (mm²) across different time points for vehicle, fasudil, ozagrel, and combination.

G: Graph comparing infarct volume (mm³) among different treatments.

H: Infarct area (mm²) in cortex and subcortex for each treatment group.

I: Infarct volume (mm³) comparison in cortex and subcortex, with vehicle, fasudil, ozagrel, and combination treatments.
Fig. 5

A

- Vehicle
- 5 min
- 3 h

Infarct area (mm²)

Coronal slice

B

Infarct volume (mm³)

Vehicle 5 min 3 h

Time after I/R

**

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Fig. 6

A

Vehicle

B

Fasudil

C

Ozagrel

D

Combination

rCBF

Before Ischemia After

Before Ischemia After

Before Ischemia After

Before Ischemia After

n.s.

n.s.

n.s.

*
Fig. 7

A

P-eNOS

β-actin

140 kDa

37 kDa

B

MCAO

Control  Vehicle  Fasudil  Ozagrel  Combination

P-eNOS

0.0  0.2  0.4  0.6  0.8  1.0  1.2  1.4  1.6
Fig. 8

A

- Vehicle
- Combination + L-NAME

B

n.s.

Infarct area (mm²)

Infarct volume (mm³)

Coronal slice

Vehicle

Combination + L-NAME
Fig. 9

- MLC
- p-MLC
- Actin

Control | HF | Ozagrel | HF + Ozagrel