Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 498 (2004) 179–188

www.elsevier.com/locate/ejphar

# Restoration of middle cerebral artery thrombosis by novel glycoprotein IIb/IIIa antagonist FK419 in guinea pig

Akira Moriguchi, Kayoko Mihara*, Toshiaki Aoki, Masashi Maeda, Nobuteru Tojo, Nobuya Matsuoka, Seitaro Mutoh

*Department of Neuroscience, Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6, Kashima, Yodogawa-ku, Osaka 532-8514, Japan*

Received 25 March 2004; received in revised form 1 July 2004; accepted 6 July 2004
Available online 12 August 2004

**Abstract**

We compared the antithrombotic efficacy of FK419 [(*S*)-2-acetylamino-3-[(*R*)-[1-[3-(piperidin-4-yl)propionyl]piperidin-3-ylcarbonyl]amino] propionic acid trihydrate], a novel nonpeptide glycoprotein IIb/IIIa antagonist, with recombinant tissue plasminogen activator (rt-PA) and other antithrombotic agents (aspirin, ozagrel, argatroban and heparin). FK419 not only inhibited ADP- and collagen-induced guinea pig platelet aggregation, but also induced disaggregation for ADP-induced aggregated platelets in vitro. In the photochemically induced middle cerebral artery thrombosis model in guinea pigs, FK419 dose-dependently shortened the time to first reperfusion and the total middle cerebral artery occlusion time and reduced ischemic brain damage and ameliorated neurological deficits measured 24 h after middle cerebral artery occlusion. Rt-PA similarly improved the middle cerebral artery patency, brain damage and neurological deficits. Neither aspirin, ozagrel, argatroban nor heparin restored the middle cerebral artery blood flow and improved the brain damage or neurological deficits. These results demonstrated that novel glycoprotein IIb/IIIa antagonist FK419 could disperse thrombus and ameliorated ischemic brain damage, suggesting that FK419 would be an attractive intervention for stroke patients.


*Keywords:* FK419; Platelet glycoprotein IIb/IIIa; Stroke; (Guinea pig); Thrombolysis

## 1. Introduction

Stroke is the major leading cause of death in most industrialized countries. Though recombinant tissue plasminogen activator (rt-PA) is approved for treating acute ischemic stroke, the utilization of this treatment is limited due to its potential risk of symptomatic brain hemorrhage and a brief 3-h time window of efficacy from symptom onset to treatment (NINDS, 1995). Furthermore, it was reported that rt-PA often fails to lyse large clots (del Zoppo et al., 1992), arteries reocclude in about a third of cases (Alexandrov and Grotta, 2002) and flow may remain stagnant in the microcirculation despite clot lysis (del Zoppo et al., 1991). Thus, alternative therapeutic approaches, including substances that are more effective than rt-PA, are desirable for acute ischemic stroke.

Antithrombotic agents such as aspirin and heparin have been commonly used in acute stroke patients. Enhanced platelet activation is observed after the onset of ischemic stroke (Koudstaal et al., 1993; van Kooten et al., 1994; Grau et al., 1998; Zeller et al., 1999). Recently, two major trials of aspirin in acute ischemic stroke (International Stroke Trial and Chinese Acute Stroke Trial) revealed that early aspirin use produces a small but definite benefit (International Stroke Trial Collaborative Group, 1997; Chinese Acute Stroke Trial (CAST) Collaborative Group, 1997; Chen et al., 2000). Thus, antiplatelet therapy might be useful in the acute ischemic stroke. In contrast, heparin has been proven ineffective in clinical studies (Bath et al., 2000). Ozagrel, a selective thromboxante $A_2$ synthase inhibitor, has been widely used in Japan for treating thrombotic or lacunar

* Corresponding author. Tel.: +81 6 6390 1153; fax: +81 6 6304 5367.
*E-mail address:* kayoko_mihara@po.fujisawa.co.jp (K. Mihara).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.07.050

stroke, and argatroban, a thrombin inhibitor, has recently been tested in clinical evaluations in acute stroke patients.

Platelet activation triggered by a variety of agonists leads to the platelet aggregation in which the final step is mediated by the binding of activated glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) with its ligand fibrinogen (Pytela et al., 1986). Therefore, inhibition of this interaction might provide potent antiplatelet effects against all agonist stimuli and better therapeutic intervention than existing anti-platelet agents such as aspirin and ozagrel. We have recently identified FK419 [(*S*)-2-acetylamino-3-[(*R*)-[1-[3-(piperidin-4-yl)propionyl]piperidin-3-ylcarbonyl]amino] propionic acid trihydrate; Fig. 1] as a novel nonpeptide selective glycoprotein IIb/IIIa antagonist (Aoki et al., 1997; Mihara et al., submitted). FK419 inhibited the binding between human fibrinogen and purified human glycoprotein IIb/IIIa receptor ($\alpha_{IIb}\beta_3$). FK419 had no affinity to vitronectin receptor ($\alpha_v\beta_3$) and fibronectin receptor ($\alpha_5\beta_1$), suggesting that FK419 is a selective glycoprotein IIb/IIIa receptor antagonist. Like other glycoprotein IIb/IIIa antagonists, FK419 showed a species specificity. FK419 inhibited platelet aggregation most potently in human and monkeys, 10 times less potent in dogs and guinea pigs and no activity in rats. Selective ligand inhibition by FK419 enabled almost complete inhibition of platelet aggregation without prolongation of bleeding time in dogs. This feature might be advantageous for the treatment of ischemic stroke, in which intracranial hemorrhage by antithrombotic agents is the most lethal complication.

A photochemical reaction between rose bengal and transluminal light irradiation leads to endothelial injury followed by platelet adhesion, aggregation and formation of occlusive platelet-rich thrombus at the irradiated site (Saniabadi et al., 1995) and is widely used to examine the effects of antithrombotic agents (Umemura et al., 1993; Nishiyama et al., 1994; Kaku et al., 1998). We have previously reported that FK419 prevented reocclusion after middle cerebral artery reperfusion and reduced ischemic brain damage in guinea pig photochemically induced thrombosis model (Moriguchi et al., 2004). Recently, thrombolytic activity of glycoprotein IIb/IIIa antagonists has been reported in coronal and femoral arteries (Mousa et al., 1994; Gold et al., 1997; Domanovits et al., 1998). However, the thrombolytic activity of glycoprotein IIb/IIIa antagonists has not been systemically evaluated well. In the present study, we compared the effects on middle cerebral artery blood flow and protective effects on the brain damage of FK419 with that of rt-PA, aspirin, ozagrel, argatroban and heparin in a guinea-pig middle cerebral artery occlusion model to clarify the therapeutic potential of FK419 for the treatment of acute ischemic stroke.

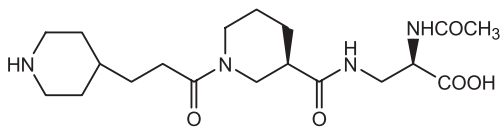


Fig. 1. Chemical structure of FK419.

## 2. Materials and methods

### 2.1. Animals

Male Hartley guinea pigs (308.2–584.5 g, SLC, Shizuoka, Japan) were used. The animals were purchased at least 1 week before the experiments. They were given free access to food and water and maintained on 12-h light/dark cycle in a controlled temperature (23±1 °C) and humidity (55±5%). All animal experimental procedures were performed under the guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical (Osaka, Japan).

### 2.2. Reagents

FK419 (anhydrate or trihydrate forms), ozagrel (anhydrate form or HCl salt) and argatroban were synthesized at Fujisawa Pharmaceutical. Heparin sodium, aspirin and rt-PA (Alteplase, Activacin®) were purchased from Shimizu Pharmaceutical (Sizuoka, Japan), Wako (Osaka, Japan) and Kyowa Hakko (Tokyo, Japan), respectively. ADP was purchased from Sigma (St. Louis, MO). Collagen was purchased from Nycomed (Munich, Germany). Isoflurane was purchased from Dainippon Pharmaceutical (Osaka, Japan). Rose bengal was obtained from Wako. All other chemicals and solvents used were commercial products and of analytical grade. FK419 were dissolved and diluted in saline for in vitro studies. For studies in vivo, FK419 was used as an anhydride and also dissolved in saline. Rt-PA was dissolved in distilled water and diluted with saline. Aspirin and heparin were dissolved and diluted in saline. An equal molar ratio of NaOH was added when ozagrel was dissolved in saline. Argatroban was dissolved in 5% glucose containing 1.1 or 1.0 M equivalent of HCl. Each drug or saline was injected (i.v. bolus of 1.5 ml/kg) followed by infusion at 5 ml/kg/h for 30 min (for rt-PA) and 3 h (for FK419, aspirin, ozagrel, argatroban and heparin).

### 2.3. Platelet aggregation and disaggregation studies in vitro

Blood was collected from the abdominal aorta into 1:10 sodium citrate (3.8%, pH 7.4) under ether anesthesia, and platelet-rich plasma was prepared by rapid centrifugation (Model 5100 with Swing-rotor RS720, Kubota, Tokyo, Japan) of whole blood at 1200 rpm for 10 min at room temperature. Platelet-poor plasma was obtained from the remaining blood by centrifugation at 3000 rpm for 10 min. The platelet aggregation assay was performed using an NBS HEMA TRACER 801 T-4A (Nikobioscience, Tokyo, Japan). Light transmittance of platelet-poor plasma was calibrated as 100%. Platelet-rich plasma was incubated for 2 min with FK419, aspirin or ozagrel in the

aggregometer at 37 °C. Agonist was added and the change in a relative light transmittance was monitored as an aggregation curve. ADP (1 or 20 μM) and collagen (1 μg/ml) were used. Aggregation percentage was measured as the maximum response during the observation period. Percentage inhibition of aggregation in drug-treated samples was determined relative to aggregation in the control sample. When drug-induced disaggregation was assessed, various concentrations of test compounds were added at 1 min after the addition of 20 μM of ADP and were monitored for 9 min after addition of the drugs. In the case of collagen (1 μg/ml)-induced aggregation, test compounds were added when the aggregation surpassed more than 60%. The maximum decrease of aggregation compared with that of when test compounds were added was calculated.

### 2.4. Coagulation time studies in vitro

Platelet-poor plasma was prepared from guinea pigs, and FK419, heparin or argatroban was added. Coagulation times were measured using an automatic Coagulometer (CA-6000, Sysmex, Kobe, Japan) by recording 50% coagulating point time by the optical dispersion light measurement system. Activated partial thromboplastin time and prothrombin time were measured up to 120 s.

### 2.5. Fibrinolysis studies in vitro

Guinea pig blood was collected from abdominal aorta, under ether anesthesia, into a plastic tube containing citrate. The blood (1.35 ml) was incubated with the drug solution (0.15 ml) for 5 min at 37 °C and then centrifuged to obtain platelet-poor plasma. To each 0.5 ml of plasma, 4.5 ml of 3.8 mM acetic acid was added and the mixture was allowed to stand for 1 h at 4 °C. After centrifugation of the acidified plasma, the precipitate was dissolved in 0.5 ml of 100 mM phosphate buffer (pH 7.4) to obtain the euglobulin solution. To each 0.4 ml of this solution, 0.08 ml of 50 IU/ml thrombin (Mochida Pharmaceutical, Tokyo, Japan) solution was added and clot lysis time was measured macroscopically.

### 2.6. Platelet aggregation studies ex vivo

Guinea pigs were anesthetized with isoflurane (2% for induction; 1% for maintenance) in a mixture of air and 30% $O_2$. A catheter was inserted into the left jugular vein for the administration of drugs. After recovery from anesthesia, FK419 or aspirin was administered until sacrifice. Blood samples were collected into 3.8% sodium citrate from the abdominal aorta under ether anesthesia 5 min, 1 or 3 h after dosing of FK419 or 1 h after dosing of aspirin. Platelet-rich plasma and platelet-poor plasma were similarly prepared and ADP (1 μM)- or collagen (1 μg/ml)-induced platelet aggregation was measured.

### 2.7. Coagulation time studies ex vivo

Sixty minutes after initiation of argatroban or heparin administration, animals were anesthetized with ether and their blood was drawn from the abdominal aorta into a plastic tube containing 3.8% sodium citrate. Platelet-poor plasma was prepared and coagulation times were measured as described above.

### 2.8. Photochemically induced middle cerebral artery occlusion in guinea pigs

The left middle cerebral artery was photochemically occluded according to the method of Moriguchi et al. (2004). Briefly, animals were anesthetized with isoflurane (2% for induction; 1% for maintenance) in a mixture of air and 30% $O_2$. A catheter for the administration of drugs or rose bengal was inserted into the left jugular vein. After a left temporal incision, the temporal muscle was removed by an electric cauterizer. A subtemporal craniotomy was performed using a dental drill under an operation microscope to open a 6-mm diameter oval bony window. The main trunk of the middle cerebral artery was observed without cutting dura matter. The head of a 3-mm diameter optic fiber mounted on a micromanipulator was placed on the middle cerebral artery segment proximal to the olfactory tract for photoirradiation. A pulsed Doppler flow probe (HHP-20; Crystal Biotech, Northboro, MA) connected to a pulsed Doppler flowmeter (PD-20; Crystal Biotech) was placed on the distal part of the middle cerebral artery to measure blood flow for 90 min. Photoirradiation was conducted using a xenon lamp (L2859-03; Hamamatsu Photonics, Hamamatsu, Japan) with a heat absorption filter and a green filter. When a stable baseline blood flow was obtained, rose bengal infusion (20 mg/kg for 6 min) and photoirradiation with green light (wavelength 540 nm, intensity 600,000 lx for 15 min) were simultaneously started. The middle cerebral artery was considered to be occluded when blood flow had completely disappeared. Each drug except for rt-PA was administered for 3 h from just after the termination of photoirradiation; rt-PA was administered for 30 min. Body temperature was maintained at 38 °C by a heating pad during the surgery and dosing. After drug administration, the skin incision was closed, and animals were allowed to recover from anesthesia. The following parameters were measured for the evaluation of middle cerebral artery blood flow during drug administration: (A) time to first reperfusion after starting the dosing (time to first reperfusion), (B) sum of time intervals during which middle cerebral artery blood flow was not detected (total occlusion time).

Animals were examined for neurological function 24 h after middle cerebral artery occlusion with a slight modification of the method of Bederson et al. (1986). Briefly, circling, forelimb paralysis, hindlimb paralysis and resistance to lateral push were scored as follows: 0 (normal), 1

(slight to moderate deficit), 2 (severe deficit). The sum of all scores represented total neurological score. After examination of neurological functions, animals were sacrificed by cardiac perfusion with saline under pentobarbital anesthesia (50 mg/kg, i.p.) and their brains were removed. The brain was coronally sectioned at pre-selected positions using a microslicer and then stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 10 min. TTC-stained sections were subsequently photographed, and nondamaged and damaged areas both in the cerebral cortex and striatum were measured by using the image analysis software (NIH image, v1.61, Bethesda, MD). The brain damage in each animal was expressed as percent of the sum of the damaged area to the sum of the whole area of cerebrum.

### 2.9. Statistical analysis

Results are expressed as mean±S.E.M. In studies in vitro, $IC_{50}$ and $EC_{50}$ values were calculated by linear regression analysis. All data obtained from in vivo studies except for coagulation time and neurological score were statistically evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Kruskal–Wallis followed by Dunnett's multiple comparison test was used for coagulation time and neurological score. A *P*-value less than 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Platelet aggregation and disaggregation studies in vitro

The inhibitory effects of FK419, aspirin and ozagrel on platelet aggregation induced with 1 and 20 μM of ADP and 1 μg/ml of collagen in guinea pig were examined. FK419 inhibited platelet aggregation concentration-dependently, with $IC_{50}$ values of 0.38±0.029, 1.04±0.031 and 0.92±0.035 μM, respectively (Fig. 2B). In contrast to FK419, aspirin only inhibited collagen-induced aggregation ($IC_{50}$ 139±17.4 μM) and did not inhibit ADP-induced aggregation at concentration up to 640 μM. Ozagrel did not inhibit all aggregation at concentrations up to 100 μM. The addition of FK419 to a suspension of aggregating platelets produced a rapid decrease in the platelet aggregation (Fig. 2). Both the rate and extent of disaggregation were dependent on the concentration of the drugs. As 1 μM of ADP resulted in the transient aggregation, 20 μM of ADP and 1 μg/ml of collagen were used in subsequent disaggregation studies. FK419

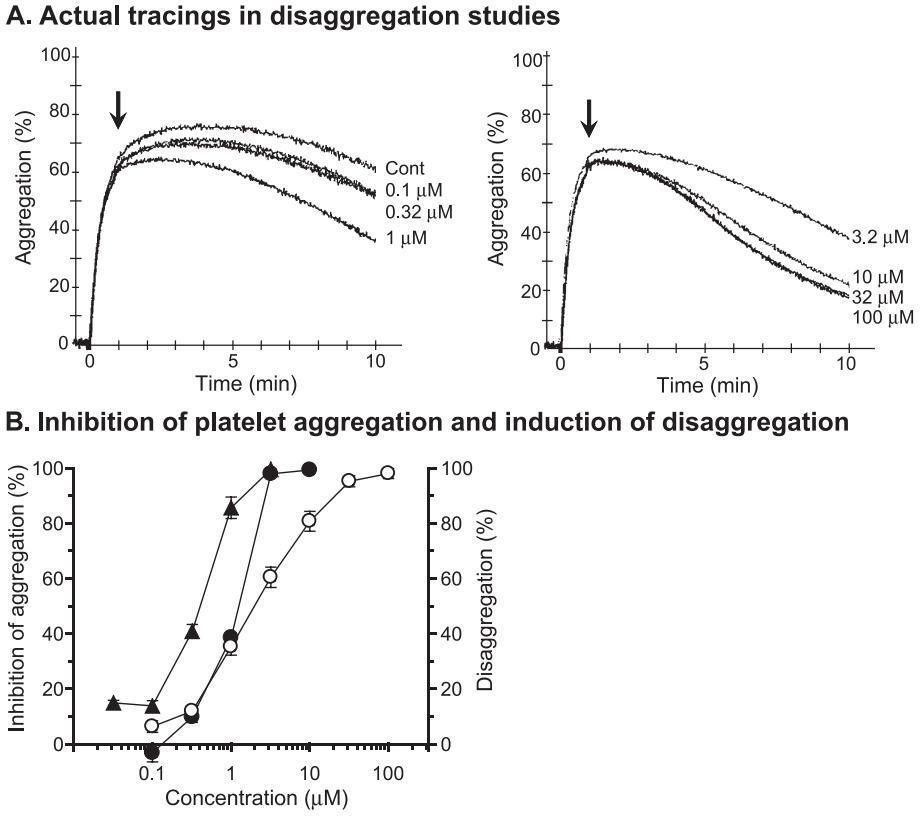


Fig. 2. Inhibition of platelet aggregation and induction of disaggregation by FK419. (A) Typical disaggregation records. FK419 was added at 1 min after addition of 20 μM ADP (arrow). (B) Dose–response curves of inhibition by FK419 on platelet aggregation (close triangle: 1 μM ADP; close circle: 20 μM ADP) and induction of disaggregation (open circle: 20 μM ADP). Each point represents the mean±S.E.M. of 5 animals for aggregation and 10 animals for disaggregation.

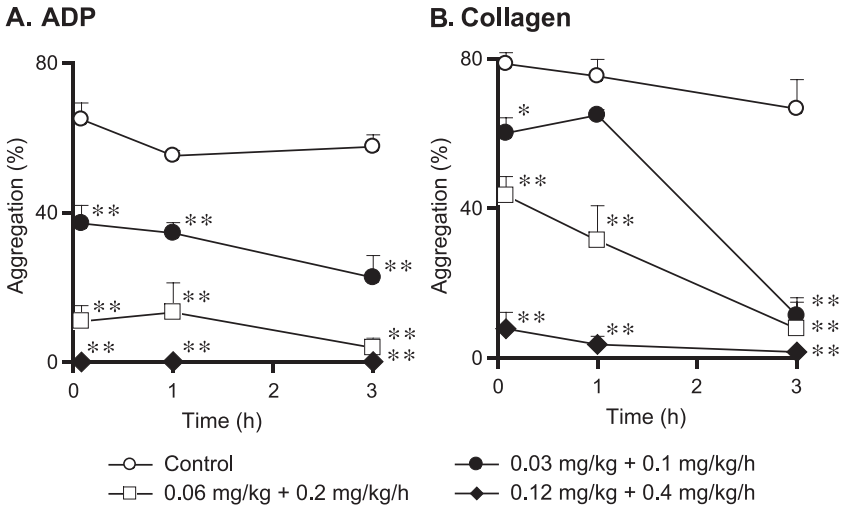


Fig. 3. Antiplatelet effects of FK419 in guinea pig. FK419 was administered to normal guinea pig. Whole blood was collected from abdominal aorta at indicated periods, and 1 μM ADP- or 1 μg/ml collagen-induced platelet aggregation was measured using platelet-rich plasma. Each point represents the mean±S.E.M. of five animals. *$P$<0.05, **$P$<0.01 versus control (one-way ANOVA followed by Dunnett's multiple comparison test).

showed concentration-dependent disaggregatory action for ADP-induced aggregation with an $EC_{50}$ value of 2.26±0.293 μM, but did not induce disaggregation for collagen-induced aggregation at concentration up to 100 μM. Aspirin and ozagrel had no disaggregatory effects on the ADP- and collagen-induced aggregations at concentration up to 640 and 100 μM, respectively.

### 3.2. Coagulation time and fibrinolysis studies in vitro

Argatroban concentration-dependently prolonged prothrombin time and activated partial thromboplastin time with doubling concentration of 0.81±0.044 and 1.29±0.059 μM, respectively. Heparin also prolonged both parameters with doubling concentration of 1.44±0.102 and 0.18±0.006 U/ml. On the other hand, FK419 had no effect on the prothrombin time and activated partial thromboplastin time at concentrations up to 100 μM. Rt-PA concentration-dependently shortened euglobulin clot lysis time with $EC_{50}$ value of 0.41±0.090 μg/ml. FK419 had no effect on the euglobulin lysis time at a concentration up to 100 μM.

### 3.3. Platelet aggregation studies ex vivo

FK419 dose-dependently inhibited ADP- and collagen-induced platelet aggregations in guinea pig ex vivo, and almost constant anti-aggregatory effect was obtained during the observation period at all doses tested (Fig. 3). Administration of FK419 at 0.03 mg/kg+0.1 mg/kg/h for 3 h yielded to about 40–50% inhibition and 0.06 mg/kg+0.2 mg/kg/h for 3 h of the drug yielded to about 70–85% inhibition, with complete inhibition achieved at 0.12 mg/kg+0.4 mg/kg/h for 3 h. Aspirin dose-dependently inhibited collagen-induced platelet aggregation. Platelets aggregation in control achieved 68±3.3%, while 47±9.7%, 5±3.0% and 2.5±1.5% aggregation were observed in the aspirin-administered animals at the dosages of 1 mg/kg +1 mg/kg/h, 3.2 mg/kg+3.2 mg/kg/h and 10 mg/kg+10 mg/kg/h for 1 h. Aspirin did not affect ADP-induced platelet aggregation.

Table 1
Effects of argatroban and heparin on coagulation times in guinea pigs

| Drug | Dose | Prothrombin time | | Activated partial thromboplastin time | |
|---|---|---|---|---|---|
| | | Time (s) | Fold | Time (s) | Fold |
| Argatroban | Control | 31±0.8 | 1.0 | 20±0.3 | 1.0 |
| | 0.9 mg/kg+3 mg/kg/h | 70±2.9 | 2.3 | 35±1.4 | 1.8 |
| | 1.8 mg/kg+6 mg/kg/h | 80±1.3* | 2.6 | 42±1.0* | 2.1 |
| Heparin | Control | 35±1.2 | 1.0 | 17±0.2 | 1.0 |
| | 10 U/kg+6 U/kg/h | 32±0.7 | 0.9 | 18±0.7 | 1.0 |
| | 100 U/kg+60 U/kg/h | 47±2.3 | 1.4 | 120±0.0** | 6.9 |
| | 1000 U/kg+600 U/kg/h | 120±0.0** | 3.5 | 120±0.0**[a] | 6.9 |

Prothrombin time and activated partial thromboplastin time were measured up to 120 s, and data which did not coagulate within 120 s are expressed as 120 s. Values are expressed as the mean±S.E.M of four or five animals.

[a] One out of four was excluded from calculation for slight coagulation.

* $P$<0.01 versus control (Kruskal–Wallis followed by Dunnett's multiple comparison test).

** $P$<0.05 versus control (Kruskal–Wallis followed by Dunnett's multiple comparison test).

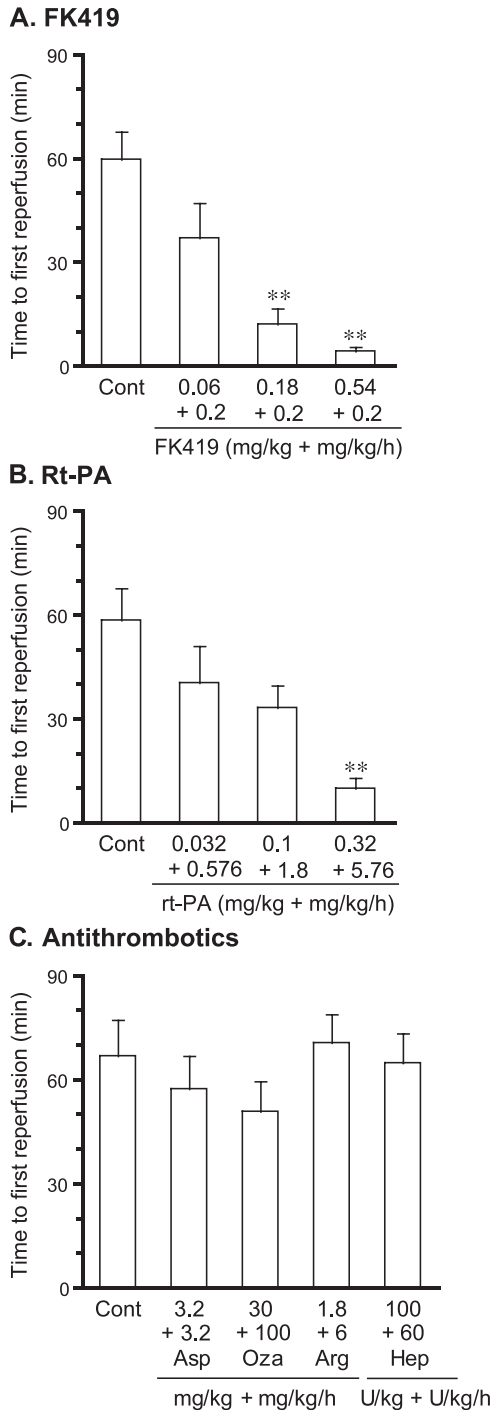


Fig. 4. Thrombolytic effects of test drugs in the guinea pig photothrombotic middle cerebral artery occlusion model. (A) FK419, (B) rt-PA and (C) aspirin (Asp), ozagrel (Oza), argatroban (Arg) and heparin (Hep) were administered from just after the end of photoirradiation for 3 h. Each column represents the mean±S.E.M. of 10 or 12 animals. **$P$<0.01 versus control (one-way ANOVA followed by Dunnett's multiple comparison test).

### 3.4. Coagulation time studies ex vivo

In argatroban- and heparin-treated groups, prothrombin time and activated partial thromboplastin time were dramatically prolonged (Table 1). Argatroban prolonged activated partial thromboplastin time and prothrombin time about 1.8–2.3-fold at 0.9 mg/kg+3 mg/kg/h for 1 h and about 2.1–2.6-fold at 1.8 mg/kg+6 mg/kg/h for 1 h. Heparin prolonged activated partial thromboplastin time from 100 U/

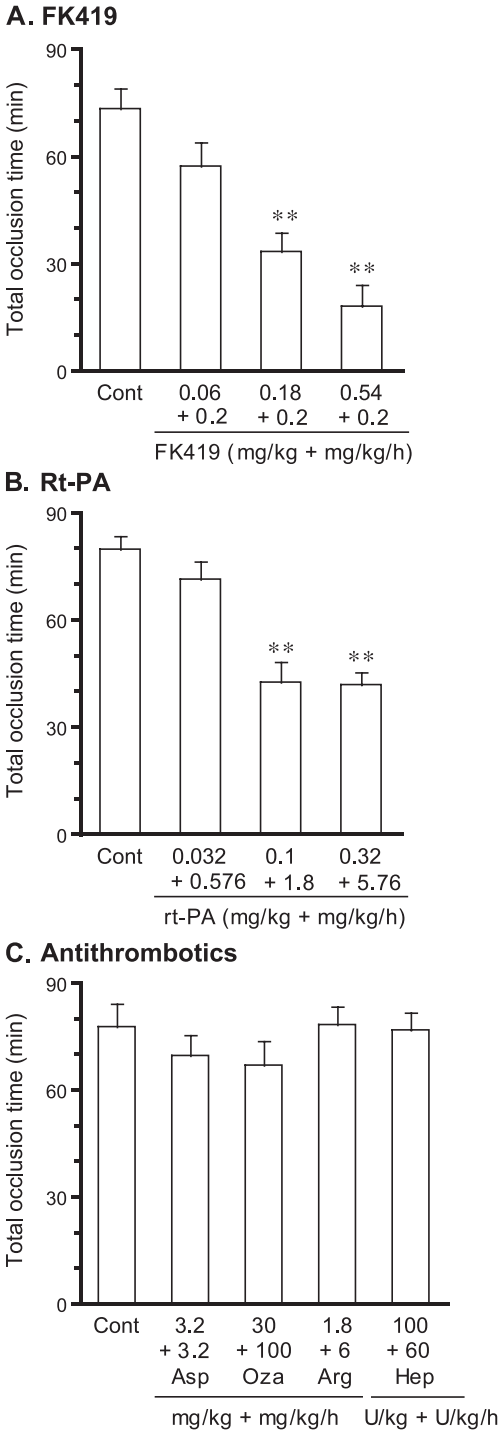


Fig. 5. Effects of test drugs on total occlusion time in the guinea pig photothrombotic middle cerebral artery occlusion model. (A) FK419, (B) rt-PA and (C) aspirin (Asp), ozagrel (Oza), argatroban (Arg) and heparin (Hep) were administered from just after the end of photoirradiation for 3 h. Each column represents the mean±S.E.M. of 10 or 12 animals. **$P$<0.01 versus control (one-way ANOVA followed by Dunnett's multiple comparison test).

Table 2
Effect of FK419, rt-PA and aspirin, ozagrel, argatroban and heparin on the neurological scores in guinea pigs 24 h after middle cerebral artery occlusion

| Drugs | Dose[a] mg/kg+mg/kg/h | Neurological score | | | | |
|---|---|---|---|---|---|---|
| | | Circling | Forelimb paralysis | Hindlimb paralysis | Resistance to lateral push | Total |
| Cont | | 1.70±0.15 | 1.40±0.16 | 1.30±0.21 | 1.20±0.13 | 5.60±0.37 |
| FK419 | 0.06+0.02 | 1.60±0.22 | 1.20±0.20 | 1.60±0.16 | 1.00±0.26 | 5.40±0.45 |
| | 0.18+0.02 | 0.70±0.26* | 0.50±0.17** | 1.30±0.15 | 0.50±0.27 | 3.00±0.52** |
| | 0.54+0.02 | 1.10±0.31 | 0.50±0.17** | 1.20±0.13 | 0.20±0.20** | 3.00±0.56** |
| Cont | | 1.70±0.15 | 1.30±0.15 | 1.90±0.10 | 1.10±0.23 | 6.00±0.37 |
| Rt-PA | 0.032+0.576 | 1.10±0.28 | 1.10±0.10 | 1.70±0.15 | 0.70±0.21 | 4.60±0.48 |
| | 0.1+1.8 | 0.80±0.29 | 1.00±0.21 | 1.10±0.18** | 0.70±0.26 | 3.60±0.58** |
| | 0.32+5.76 | 0.80±0.29 | 0.70±0.26 | 1.60±0.22 | 0.40±0.22 | 3.50±0.50** |
| Cont | | 1.75±0.13 | 1.50±0.15 | 1.50±0.15 | 1.83±0.11 | 6.58±0.36 |
| Aspirin | 3.2+3.2 | 1.92±0.08 | 1.67±0.14 | 1.50±0.15 | 2.00±0.00 | 7.08±0.19 |
| Ozagrel | 30+100 | 1.83±0.11 | 1.42±0.15 | 1.17±0.11 | 1.75±0.13 | 6.17±0.32 |
| Argatroban | 1.8+5 | 2.00±0.00 | 1.67±0.14 | 1.50±0.15 | 1.75±0.13 | 6.92±0.26 |
| Heparin | 100 U/kg+60 U/kg/h | 1.82±0.12 | 1.36±0.15 | 1.27±0.14 | 1.73±0.14 | 6.18±0.35 |

Values are expressed as the mean±S.E.M of 10–12 animals.
[a] Values mean bolus plus infusion doses and the units were mg/kg plus mg/kg/h except heparin whose units were U/kg plus U/kg/h.
* $P<0.05$ versus control (Kruskal–Wallis followed by Dunnett's multiple comparison test).
** $P<0.01$ versus control (Kruskal–Wallis followed by Dunnett's multiple comparison test).

kg+60 U/kg/h for 1 h and prothrombin time at 1000 U/kg+600 U/kg/h for 1 h.

### 3.5. Thrombotic middle cerebral artery occlusion model in guinea pigs

#### 3.5.1. Middle cerebral artery blood flow

Middle cerebral artery blood flow decreased to zero approximately 5 min after photoirradiation in all groups. Surgical microscopy revealed that the lumen of middle cerebral artery was occluded by platelet-rich thrombus at the irradiated site. Spontaneous reperfusion following the primary occlusion was monitored for 90 min after end of photoirradiation. Most of the animals showed cyclical reocclusion and reperfusion of the middle cerebral artery which is known as cyclic flow reductions (Fig. 4A). FK419 and rt-PA dose-dependently reduced the time to first reperfusion, suggesting that the drugs have thrombolytic action (Fig. 4). Significant improvements were obtained from middle dose of FK419 and at high dose of rt-PA. FK419 and rt-PA also significantly shortened total occlusion time (Fig. 5). Total occlusion time decreased in FK419-treated animals more dramatically compared to rt-PA-treated animals, indicating that FK419 reduced the numbers and duration of reocclusion more effectively (Fig. 5). Aspirin, ozagrel, argatroban and heparin were also examined. The dose of aspirin used was chosen at which aspirin almost completely inhibited collagen-induced platelet aggregation ex vivo. The dose of ozagrel was decided from the previous study at which ozagrel inhibited thromboxane B2 production (Moriguchi et al., 2004). The doses of argatroban and heparin were chosen at which they dramatically prolonged coagulation times ex vivo. Nevertheless, none of these drugs affected the time to first reperfusion or total occlusion time (Figs. 4 and 5).

#### 3.5.2. Neurological scores

Neurological scores were dose-dependently improved by FK419 and rt-PA treatment (Table 2). Statistically significant improvements for total score were observed at the middle and the high doses of FK419 and rt-PA. Aspirin, ozagrel, argatroban or heparin did not ameliorate neurological score. An animal died in heparin-treated group.

#### 3.5.3. Ischemic brain damage

FK419 and rt-PA reduced ischemic brain damage in a dose-dependent fashion (Fig. 6). Significant effects in total (cerebral cortex+striatum) or cerebral cortex were observed from low dose of FK419 and middle dose of rt-PA. Percent reductions in ischemic brain damage in cerebral cortex by FK419 and rt-PA were as follows: low doses, 33% and 3%; middle doses, 59% and 26%; high doses, 57% and 41%, respectively. Aspirin, ozagrel, argatroban and heparin did not affect brain damage (Fig. 6).

## 4. Discussion

We examined the thrombolytic efficacy of a novel glycoprotein IIb/IIIa antagonist, FK419, in comparison with rt-PA, aspirin, ozagrel, argatroban and heparin using a guinea pig photochemically induced middle cerebral artery thrombosis model. We had previously reported that FK419 dose-dependently prevented the reocclusion of middle cerebral artery after recanalization under milder occlusion conditions (Moriguchi et al., 2004). Time to first reperfusion and duration of cyclic flow reductions were increased when longer photoirradiation was used to induce severe endothelial injury (Kawano et al., 1998). As the purpose of the present study was to determine the effects of FK419 on the initial obstructive thrombus, the time of photoirradiation in this study was prolonged from 10 to 15 min to make more

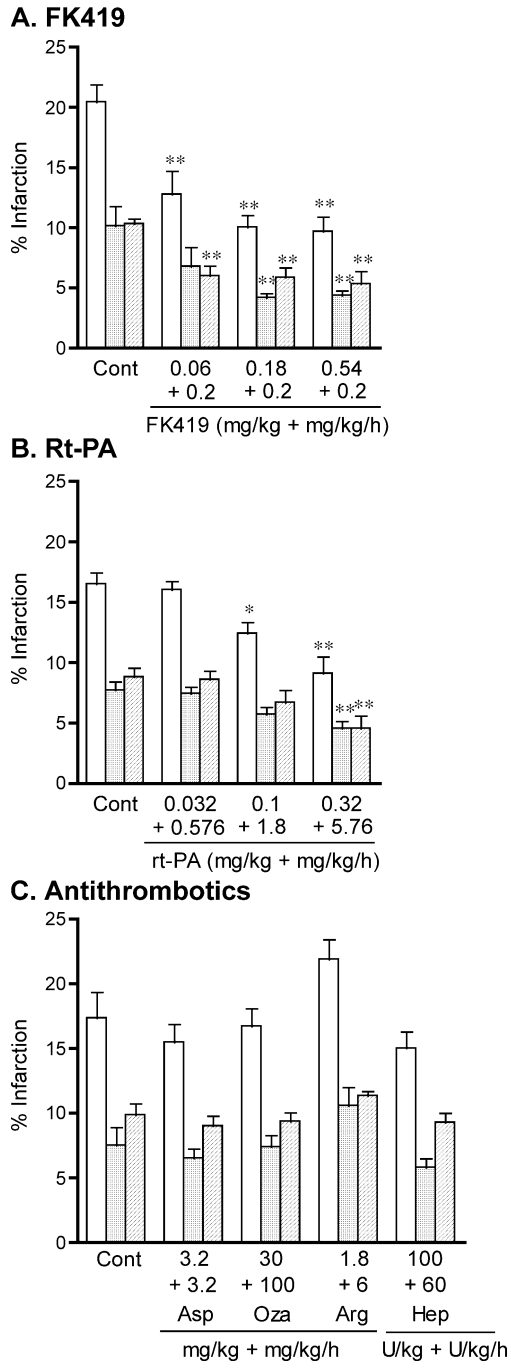


Fig. 6. Effect of (A) FK419, (B) rt-PA and (C) aspirin (Asp), ozagrel (Oza), argatroban (Arg) and heparin (Hep) on the brain damage in guinea pigs 24 h after middle cerebral artery occlusion. Total (open), cortical (dotted) and subcortical (hatched) brain damages were measured by TTC staining. Each column represents the mean±S.E.M. of 10–12 animals. *$P$<0.05, **$P$<0.01 versus control (one-way ANOVA followed by Dunnett's multiple comparison test).

stable thrombus. The time to first reperfusion in the control group was prolonged to approximately 60 min (whereas it was 16 min under the condition for 10 min of photo-irradiation), and % infarction of the brain damage had a good correlation with the time to first reperfusion. Assuming that the initial plasma concentrations of the drug might be important for the thrombolytic action, the bolus dosages were increased while maintaining a constant infusion dosage. The lowest dose of FK419 examined in the present study was determined as a minimum effective dose to prevent reocclusion in our previous study (Moriguchi et al., 2004). Here we show that FK419 dose-dependently decreases the time to first reperfusion, suggesting that FK419 effectively lysed the obstructive thrombus in middle cerebral artery, though FK419 had no effects on coagulation and fibrinolysis cascade. Furthermore, FK419 dose-dependently ameliorated brain infarction and neurological deficits assessed at 24 h after the ischemic insults.

Rt-PA and other antithrombotic agents were also evaluated under similar experimental conditions. Rt-PA was administered as 10% bolus and 90% infusion for 30 min, rather than for 60 min, to ensure detection of the thrombolytic action of rt-PA clearly. Rt-PA dose-dependently lysed the middle cerebral artery thrombus and improved cerebral infarction and neurological deficits, suggesting that fibrin, in addition to activated platelets, could be involved in the formation of the thrombus in this model. The effective dosages of rt-PA were similar to that previously reported (Nishiyama et al., 1994).

In contrast to FK419 and rt-PA, other antithrombotic agents hardly showed therapeutic efficacy in this model. Insufficient dose levels were unlikely since the ratio of bolus and infusion dosage of each drug was chosen so that drug can quickly reach a constant plasma concentration, and sufficient dosing was actually confirmed by ex vivo studies. While aspirin almost completely inhibited ex vivo collagen-induced platelet aggregation and ozagrel significantly inhibited thromboxane $B_2$ production (Moriguchi et al., 2004), they minimally affected middle cerebral artery blood flow. Poor efficacy of aspirin is in good accordance with most of the reports in which it was administered even before occlusion (Kawano et al., 1999). The limited efficacy of these agents might be related to the fact that they inhibit only one specific pathway of platelet activation and that they could not disaggregate the aggregated platelets. Argatroban and heparin scarcely affected middle cerebral artery blood flow and did not reduce infarction, despite the fact that the same dose of argatroban and heparin prolonged coagulation parameters. The effect of argatroban on arterial thrombosis is somewhat controversial. Argatroban was reported to ameliorate ischemic brain damage in rat thrombotic (Kawai et al., 1996) and embolic models (Morris et al., 2001), with prolongation of coagulation parameters. However, argatroban did not ameliorate brain damage 24 h after photochemical middle cerebral artery occlusion despite reducing microthrombi formation for first several hours following the occlusion (Kawai et al., 1995). Argatroban had no effect on photochemically induced thrombus formation at the site of endothelial damage in guinea pigs (Hirata et al., 1993). Low efficacy of heparin in photochemically induced thrombosis model was reported (Zhao et al., 2001; Arii et al., 2002). Considering the fact that the

doses of argatroban and heparin examined in the present study were high enough to prolong the coagulation parameters, the inhibition of coagulation cascade hardly affects the thrombolysis in guinea pig arterial thrombosis induced by endothelial damage.

Thrombolytic effect of glycoprotein IIb/IIIa antagonists have been reported in coronal and femoral arteries (Mousa et al., 1994; Gold et al., 1997; Domanovits et al., 1998) and the mechanism of which is supposed to relate to platelet disaggregation (Mousa et al., 1994; Marciniak et al., 2002) and inhibition of plasminogen activator inhibitor-1 (PAI-1) secretion (Tsao et al., 1997). These mechanisms could be involved in the action of FK419. Though plasma concentration of FK419 was not measured, it might reach the concentrations that are sufficient for its disaggregatory action achieved at effective bolus dosages estimated from ex vivo platelet inhibition studies. The fact that aspirin and ozagrel, which had no disaggregatory actions, did not show the thrombolytic effect also supports the importance of disaggregatory action of FK419. While disaggregatory action of FK419 was observed in ADP-induced, but not in collagen-induced, platelet aggregation in vitro, FK419 shortened the time to first reperfusion in middle cerebral artery thrombosis model, suggesting that obstructive thrombus is more responsible to glycoprotein IIb/IIIa antagonists in vivo than in vitro. FK419 dose-dependently inhibited secretion of PAI-1 from human activated platelet at similar concentrations as platelet aggregation in vitro, whereas aspirin did not (unpublished observation). Though inhibition of PAI-1 secretion was not confirmed in guinea pig platelets, inhibition of PAI-1 release could be involved in its beneficial effects in this stroke model. The recanalization promoting efficacy of glycoprotein IIb/IIIa antagonist was also observed in rat embolic stroke model (Yang et al., 2001). Rt-PA has been well known as a thrombolytic agent by induction of proteolytical cleavage of fibrin which is one of the major components of thrombus. On the other hand, though glycoprotein IIb/IIIa receptor antagonists have been generally known as a potent inhibitor for platelet aggregation, they also disperse the aggregated platelets, which is another major component of thrombus, by displacing fibrinogen binding to glycoprotein IIb/IIIa receptor. As FK419 showed the beneficial thrombolytic effect in the stroke model by which the mechanism is independent of fibrinolytic action of rt-PA, FK419 could be a new and additional therapeutic agent for ischemic stroke patients.

We have previously reported that FK419 ameliorated the brain damages by preventing reocclusion after recanalization (Moriguchi et al., 2004). In the present study, FK419 prevented the reocclusion, while we did not measure the middle cerebral artery blood flow long enough. In contrast, though rt-PA induced rapid recanalization, longer duration and larger numbers of cyclic flow reductions were occurred compared to FK419. Thus, FK419 not only lysed obstructive thrombus, but also prevented the reocclusion after recanalization. This activity of preventing reocclusion might contribute to the infarction-reducing efficacy observed in a low-dose group. It was reported that reocclusion was occurred in some patients treated with rt-PA (Alexandrov and Grotta, 2002). Therefore, FK419 is a promising agent with thrombolytic and antithrombotic efficacies. Furthermore, a combination therapy of FK419 together with rt-PA might be a more likely suggestion for intervention in ischemic stroke.

In conclusion, we demonstrate that FK419 effectively restores the middle cerebral artery blood flow by dispersing the obstructive thrombus and ameliorated neurological outcome and ischemic brain damage as well as rt-PA. These findings suggest that FK419 would be an attractive intervention as the treatment of acute ischemic stroke.

## Acknowledgments

The authors would like to thank Dr. Raymond D. Price for his helpful comments in preparing the manuscript.

## References

Alexandrov, A.V., Grotta, J.C., 2002. Arterial reocclusion in stroke patients treated with intravenous tissue plasminogen activator. Neurology 59, 862–867.

Aoki, T., Senzaki, K., Honda, S., Tomiyama, Y., Okubo, M., Takahashi, F., Seki, J., 1997. FR189419 is a novel αIIbβ3 antagonist with a minimum adverse effect on bleeding time. Thromb. Haemost. 77, 666 (Suppl.).

Arii, K., Igarashi, H., Arii, T., Katayama, Y., 2002. The effect of ozagrel sodium on photochemical thrombosis in rat: therapeutic window and combined therapy with heparin sodium. Life Sci. 71, 2983–2994.

Bath, P.M., Iddenden, R., Bath, F.J., 2000. Low molecular weight heparins and heparinoids in acute ischemic stroke: a meta-analysis of randomized controlled trials. Stroke 31, 1770–1778.

Bederson, J.B., Pitts, L.H., Tsuji, M., Nishimura, M.C., Davis, R., Bartkowski, H., 1986. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 17, 472–476.

Chinese Acute Stroke Trial (CAST) Collaborative Group, 1997. CAST: randomised placebo-controlled trial of early aspirin use in 20000 patients with acute ischaemic stroke. Lancet 349, 1641–1649.

Chen, Z.M., Sandercock, P., Pan, H.C., Counsell, C., Collins, R., Liu, L.S., Xie, J.X., Warlow, C., Peto, R., 2000. Indications for early aspirin use in acute ischemic stroke: a combined analysis of 40 000 randomized patients from the Chinese acute stroke trial and the international stroke trial. On behalf of the CAST and IST collaborative groups. Stroke 31, 1240–1249.

del Zoppo, G.J., Schmid-Schönbein, G.W., Mori, E., Copeland, B.R., Chang, C.M., 1991. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke 22, 1276–1283.

del Zoppo, G.J., Poeck, K., Pessin, M.S., Wolpert, S.M., Furlan, A.J., Ferbert, A., Alberts, M.J., Zivin, J.A., Wechsler, L., Busse, O., Greenlee Jr., R., Brass, L., Mohr, J.P., Feldmann, E., Hacke, W., Kase, C.S., Biller, J., Gress, D., Otis, S.M., 1992. Recombinant tissue plasminogen activator in acute thrombotic and embolic stroke. Ann. Neurol. 32, 78–86.

Domanovits, H., Nikfardjam, M., Janata, K., Hornykewycz, S., Maurer, G., Laggner, A.N., Huber, K., 1998. Restoration of coronary blood flow by single bolus injection of the GPIIb/IIIa receptor antagonist c7E3 Fab in

a patient with acute myocardial infarction of recent onset. Clin. Cardiol. 21, 525–528.

Gold, H.K., Garabedian, H.D., Dinsmore, R.E., Guerrero, L.J., Cigarroa, J.E., Palacios, I.F., Leinbach, R.C., 1997. Restoration of coronary flow in myocardial infarction by intravenous chimeric 7E3 antibody without exogenous plasminogen activators. Circulation 95, 1755–1759.

Grau, A.J., Ruf, A., Vogt, A., Lichy, C., Buggle, F., Patscheke, H., Hacke, W., 1998. Increased fraction of circulating activated platelets in acute and previous cerebrovascular ischemia. Thromb. Haemost. 80, 298–301.

Hirata, Y., Takiguchi, Y., Wada, K., Matsuno, H., Umemura, K., Uematsu, T., Nakashima, M., 1993. Roles of platelet-activating factor, thromboxane $A_2$, ADP and thrombin in thrombogenesis in the guinea pig. Eur. J. Pharmacol. 231, 421–425.

International Stroke Trial Collaborative Group, 1997. The international stroke trial (IST): a randomised trial of aspirin, subcutaneous heparin, both, or neither among 19 435 patients with acute ischaemic stroke. Lancet 349, 1569–1581.

Kaku, S., Umemura, K., Mizuno, A., Yano, S., Suzuki, K., Kawasaki, T., Nakashima, M., 1998. Evaluation of a GPIIb/IIIa antagonist YM337 in a primate model of middle cerebral artery thrombosis. Eur. J. Pharmacol. 345, 185–192.

Kawai, H., Umemura, K., Nakashima, M., 1995. Effects of argatroban on microthrombi formation and brain damage in the rat middle cerebral artery thrombosis model. Jpn. J. Pharmacol. 69, 143–148.

Kawai, H., Yuki, S., Sugimoto, J., Tamao, Y., 1996. Effects of a thrombin inhibitor, argatroban, on ischemic brain damage in the rat distal middle cerebral artery occlusion model. J. Pharmacol. Exp. Ther. 278, 780–785.

Kawano, K., Ikeda, Y., Kondo, K., Umemura, K., 1998. Increased cerebral infarction by cyclic flow reductions: studies in the guinea pig MCA thrombosis model. Am. J. Physiol. 275, R1578–R1583.

Kawano, K., Ikeda, Y., Kondo, K., Umemura, K., 1999. Superiority of platelet integrin GPIIb–IIIa receptor antagonist over aspirin in preventing cyclic flow reductions in the guinea pig middle cerebral artery. Eur. J. Pharmacol. 374, 377–385.

Koudstaal, P.J., Ciabattoni, G., van Gijn, J., Nieuwenhuis, H.K., de Groot, P.G., Sixma, J.J., Patrono, C., 1993. Increased thromboxane biosynthesis in patients with acute cerebral ischemia. Stroke 24, 219–223.

Marciniak Jr., S.J., Nascekku, M.A., Furman, M.K., Michelson, A.D., Jakubowski, J.A., Jordan, R.E., Marchese, P.J., Frelinger, A.L., 2002. An additional mechanism of action of abciximab: dispersal of newly formed platelet aggregates. Thromb. Haemost. 87, 1020–1025.

Moriguchi, A., Aoki, T., Mihara, K., Tojo, N., Matsuoka, N., Mutoh, S., 2004. Antithrombotic effects of FK419, a novel nonpeptide platelet GPIIb/IIIa antagonist, in a guinea pig photochemically induced middle cerebral artery thrombosis model: comparison with ozagrel and argatroban. J. Pharmacol. Exp. Ther. 308, 1094–1101.

Morris, D.C., Zhang, L., Zhang, Z.G., Lu, M., Berens, K.L., Brown, P.M., Chopp, M., 2001. Extension of the therapeutic window for recombinant tissue plasminogen activator with argatroban in a rat model of embolic stroke. Stroke 32, 2635–2640.

Mousa, S.A., Forsythe, M.S., Diemer, M., Bozarth, J.M., Reilly, T.M., 1994. Thrombolytic and antithrombotic efficacy of the platelet GPIIb–IIIa antagonist DMP728. Coron. Artery Dis. 5, 919–927.

Nishiyama, H., Umemura, K., Saniabadi, A.R., Takiguchi, Y., Uematsu, T., Nakashima, M., 1994. Enhancement of thrombolytic efficacy of tissue-type plasminogen activator by adjuvants in the guinea pig thrombosis model. Eur. J. Pharmacol. 264, 191–198.

Pytela, R., Pierschbacher, M.D., Ginsberg, M.H., Plow, E.F., Ruoslahti, E., 1986. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. Science 231, 1559–1562.

Saniabadi, A.R., Umemura, K., Matsumoto, N., Sakuma, S., Nakashima, M., 1995. Vessel wall injury and arterial thrombosis induced by a photochemical reaction. Thromb. Haemost. 73, 868–872.

The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995. Tissue plasminogen activator for acute ischemic stroke. N. Engl. J. Med. 333, 1581–1587.

Tsao, P.W., Forsythe, M.S., Mousa, S.A., 1997. Dissociation between the anti-aggregatory and anti-secretory effects of platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) antagonists, c7E3 and DMP728. Thromb. Res. 88, 137–146.

Umemura, K., Wada, K., Uematsu, T., Nakashima, M., 1993. Evaluation of the combination of a tissue-type plasminogen activator, SUN9216, and a thromboxane $A_2$ receptor antagonist, vapiprost, in a rat middle cerebral artery thrombosis model. Stroke 24, 1077–1082.

van Kooten, F., Ciabattoni, G., Patrono, C., Schmitz, P.I.M., van Gijn, J., Koudstaal, P.J., 1994. Evidence for episodic platelet activation in acute ischemic stroke. Stroke 25, 278–281.

Yang, Y., Li, Q., Nakada, M.T., Yang, T., Shuaib, A., 2001. Angiographic evaluation of middle cerebral artery reperfusion caused by platelet glycoprotein IIb/IIIa receptor complex antagonist murine 7E3 $F(ab')_2$ in a model of focal cerebral ischemia in rats. J. Neurosurg. 94, 582–588.

Zeller, J.A., Tschoepe, D., Kessler, C., 1999. Circulating platelets show increased activation in patients with acute cerebral ischemia. Thromb. Haemost. 81, 373–377.

Zhao, B.-Q., Suzuki, Y., Kondo, K., Kawano, K., Ikeda, Y., Umemura, K., 2001. Cerebral hemorrhage due to heparin limits its neuroprotective effects: studies in a rabbit model of photothrombotic middle cerebral artery occlusion. Brain Res. 902, 30–39.