Combined effects of a factor Xa inhibitor YM466 and a GPIIb/IIIa antagonist YM128 on thrombosis and neointima formation in mice

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Summary

Thrombosis and neointima formation limit the efficacy of coronary angioplasty. Factor Xa inhibitors and GPIIb/IIIa antagonists have shown to be effective on acute thrombosis and late neointima formation, however; their combined effects remain to be elucidated. Vascular injury was induced by FeCl₃ in the carotid artery in mice. For thrombosis studies, the test drug was orally administered 1 hour before vascular injury. For neointima studies, the test drug was orally administered 1 hour before and twice daily for 1 week after vascular injury, and then histological analysis was performed 3 weeks after vascular injury. YM466 inhibited thrombotic occlusion at 30 mg/kg with prolongation of prothrombin time (PT), and tail transection bleeding time (BT) was affected at 100 mg/kg. YM466 also inhibited neointima formation at 10 mg/kg. YM128 inhibited thrombotic occlusion and neointima formation at 10 and 30 mg/kg, respectively, with inhibition of platelet aggregation and prolongation of BT. In contrast, the combination of 10 mg/kg YM466 and 3 mg/kg YM128 inhibited thrombotic occlusion and neointima formation without affecting PT, platelet aggregation and BT. Concomitant inhibition of factor Xa and GPIIb/IIIa may provide a safer and more effective therapeutic regimen for treatment of coronary angioplasty.

Keywords

Combination, factor Xa inhibitor, GPIIb/IIIa antagonist, thrombosis, neointima formation

Introduction

Despite the high primary success rate of percutaneous transluminal coronary angioplasty, acute thrombosis and late restenosis still limit the benefits of this procedure. Recently, successful multiple pharmacological approaches to prevent thrombosis and neointima formation have been reported (1), and several promising agents have been launched or are in clinical trials (2).

The activated serine protease factor X (factor Xa) is the key enzyme at the convergent point of the intrinsic and extrinsic coagulation pathways. Since one molecule of factor Xa can generate 138 molecules of thrombin in 1 minute, the anticoagulant effects can be expected to be more efficient by inhibiting factor Xa rather than thrombin (3). Moreover, the risk of bleeding is expected to decrease, because factor Xa inhibitors specifically affect coagulation but do not affect platelet function. In addition to its important role in the coagulation system, factor Xa can initiate receptor-mediated intracellular signal transduction pathways and functional responses via the reaction with the effector cell protease receptor-1 (EPR-1), which is known as a specific cell membrane receptor for factor Xa that is found on leukocytes, platelets, vascular endothelial cells and smooth
muscle cells (4). Through its interaction with EPR-1, factor Xa may function as a mediator of inflammation, and may also contribute to the pathogenesis of neointima formation after vascular injury. Several factor Xa inhibitors have exerted antithrombotic effects in thrombosis models (5, 6) and have inhibited neointima formation in vascular injury models (7, 8). At the present time, several factor Xa inhibitors are under clinical trials for patients with acute coronary syndrome (ACS) (9) and deep venous thrombosis after orthopedic surgery (10).

On the other hand, platelets play an important role not only in acute thrombosis but also in the development of neointima formation since platelets provide several kinds of stimulation factors, such as platelet-derived growth factor and transforming growth factor-β, leading to migration and proliferation of smooth muscle cells (11). The platelet GPIIb/IIIa mediates binding of activated platelets to fibrinogen, the final common pathway of aggregation. Several GPIIb/IIIa antagonists have exerted antithrombotic effects in thrombosis models (12) and have inhibited neointima formation in angioplasty models (13), and are now available for patients with ACS (14, 15). Although the GPIIb/IIIa antagonist abciximab showed a reduction both in ischemic complications and clinical restenosis in the early trial (16), recent large-scale, placebo-controlled, randomized trials of abciximab and other GPIIb/IIIa antagonists have failed to provide commensurate reductions in late composite ischemic endpoints, despite the potent inhibition of platelet aggregation (17, 18).

Recently, we discovered a potent and specific factor Xa inhibitor, YM466 (19). The Ki values of YM466 for human factor Xa and thrombin were 1.3 nM and over 100 μM, respectively. Furthermore, we also discovered a potent GPIIb/IIIa antagonist, YM-57029 (20), and its prodrug YM128 (21). YM-57029 inhibited fibrinogen binding to GPIIb/IIIa about 1,000-fold more potently than RGDS, but had no effect on vitronectin binding to αvβ3 and αvβ5, or fibronectin binding to αvβ5. Although we have reported the antithrombotic effects of YM466 and YM-57029 in several thrombosis models, their combined effects have not been studied so far (20, 22). In this study, we investigated the combined effects of YM466 and YM128 on thrombosis and neointima formation model in mice. Based on our knowledge and research, this is the first report to show the effects of a factor Xa inhibitor combined with a GPIIb/IIIa antagonist on thrombosis and neointima formation after vascular injury.

**Materials and methods**

**Agents**
YM466 (19), YM-57029 (20) and YM128 (21) were synthesized at Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan). Adenosine diphosphate (ADP) was purchased from MC Medical (Tokyo, Japan). Other reagents were commercially obtained.

**Animals**
Male ICR mice weighing 35-45 g were purchased from Japan SLC (Shizuoka, Japan). The animals were allowed free access to standard mice food and water. All animal experiments were performed in accordance with the regulations of the Animal Ethics Committee of Yamanouchi Pharmaceutical Co., Ltd.

**In vitro coagulation and platelet aggregation**
Platelet-poor plasma (PPP) and platelet-rich plasma (PRP) were prepared from citrate-anticoagulated blood (3.2% sodium citrate: blood = 1:9) collected from the vena cava of fasted mice anesthetized with urethane (1.4 g/kg i.p.). PRP was obtained by centrifugation at 200 g for 5 minutes at room temperature. This residue was centrifugated further at 2,000 g for 10 minutes to obtain PPP. Prothrombin time (PT) in PPP was measured using a coagulometer (KC10A; Amelung Co., Lehbrinksweg, Germany) as follows: 40 μl of plasma plus 10 μl of a test sample was incubated at 37°C for 1 minute and then mixed with 50 μl of the PT reagent (Orthobrain Thromboplastin, Ortho Diagnostic Systems Co., Tokyo, Japan). Platelet aggregation in PRP was measured using an aggregometer (MCM Hema Tracer 212, MC Medical, Tokyo, Japan) by recording the increase in light transmission through a stirred suspension maintained at 37°C for 5 minutes. For *in vitro* platelet aggregation studies, YM-57029, an active metabolite of YM128, was used. Platelet aggregation in 80 μl of PRP (3 x 10^9/μl) plus 10 μl of test sample was induced by 10 μl of ADP (20 μM). Inhibitory activity was calculated by dividing the maximum rate of decrease in absorbance of a mixture containing the test sample by the maximum rate obtained in the presence of a single buffer.

**Ex vivo coagulation and platelet aggregation**
YM466 and YM128, suspended in 0.5% methylcellulose, were orally administered to fasted mice. One hour after administration, blood was collected, and then PPP and PRP were prepared to measure PT and platelet aggregation as described above.

**FeCl3-induced arterial thrombosis**
A vascular injury-induced thrombosis model was used according to the method of Fay et al. (23). Fasted mice were anesthetized by i.p. injection of urethane at 1.4 g/kg. The left carotid artery was carefully exposed through a midline cervical incision. Then, a pulse Doppler flow probe (diameter: 0.5 mm, DBF05S; Primetech Co., Tokyo, Japan) was placed around the carotid artery. One hour after oral administration of the test drug, a filter paper (1 mm × 1 mm) saturated with 10% FeCl3 solution was applied to the exposed carotid artery proximal to the probe for 3 minutes. Carotid blood flow was monitored and recorded using a Doppler blood flow velocimeter (PVD-20; Crystal Biotech America, Hopkinton, MA, USA) and a polygraph (WI-681G, Nihon Kohden, Tokyo, Japan) for 30 minutes thereafter. The time at which the flow velocity decreased...
to zero was recorded as time to occlusion of the vessel. If the carotid artery was not occluded within 30 minutes, time to occlusion was defined as 30 minutes for statistical analyses.

**Tail transection bleeding time**
YM466 and YM128 were orally administered to fasted mice. One hour after administration, tail tips, 1 mm from the distal ends, were surgically removed with a scalpel after anesthesia with urethane (1.4 g/kg i.p.). Blood flowing from the incision was gently wiped away with a filter paper every 30 seconds. BT was measured as time elapsed until bleeding stopped. When bleeding lasted longer than 30 minutes, the BT was recorded as 30 minutes for statistical analyses.

**FeCl₃-induced neointima formation**
Fasted mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.). Atropine sulfate (0.6 mg/kg) was also injected subcutaneously. One hour after oral administration of the test drug, the left carotid artery was exposed to FeCl₃ for 3 minutes, as described above. The animals were allowed to recover and were followed for 3 weeks. The test drug was orally administered twice daily (12 hour intervals) for 1 week after the surgical operation. Animals were re-anesthetized with sodium pentobarbital and perfusion fixation under physiological pressure was performed for 10 minutes with 4% formalin in PBS after the left ventricular cardiac puncture. An injured segment (approximately 2 mm in length, including an injured vessel) of the left carotid artery, which can be easily distinguished due to discoloration because of FeCl₃ injury, and a normal segment of the contralateral artery were carefully dissected, postfixed overnight at room temperature, and embedded in paraffin.

Morphometric analysis was performed on carotid artery cross-sections in a blinded manner via a microscope equipped with an NIH image system. A series of cross-sections (6 μm) of the artery were stained with hematoxylin and eosin (H&E), and the area within the internal elastic lamina (IEL), and the luminal area were determined in sections 24 μm apart (10 to 12 sections/slide glass, 6 slide glasses/artery). In this way, 60 to 72 sections were analyzed per artery. Quantitative analysis was done on those cross-sections with the highest degree of smooth muscle cell proliferation. The media area was defined as the area delineated by the internal and external elastic lamina. The intima was defined as the area within the IEL occupied by a neointima. The ratio of intima to media area (I/M ratio) was calculated. Luminal stenosis (expressed as a percentage) was calculated according to the following formula: intimal area/area within IEL × 100. Ferric iron deposits were detected by using Prussian blue staining according to Perls’ reaction. In brief, deparaffinized sections with 6 μm thick were stained for 20 minutes in a solution of 1% potassium hexacyanoferrate and 2% HCl (v/v), and nuclei were counterstained with nuclear fast red. The expression of α-smooth muscle actin was assessed by using the immunohistochemistry ABC method with the monoclonal antibody against the antigen (clone 1A4, Dako), and hematoxyline was used for counterstaining.

**Statistical analysis**
For coagulation study, platelet aggregation study and neointima study, data are expressed as mean ± SEM. In the thrombosis and bleeding time studies, individual data of the animal and median value of the group are indicated. Statistical analyses for time to occlusion and bleeding time were performed using the Kruskal-Wallis H-test, with Steel’s test. Statistical analysis for occlusion rate was performed using the χ²-test. Statistical analysis for morphological analyses in the neointima study was performed using Student’s t-test or Dunnnett’s multiple comparison test. A value of P < 0.05 was considered significant.

**Results**

**In vitro coagulation and platelet aggregation**
YM466 concentration-dependently prolonged PT in mice PPP, with a doubling concentration of 0.80 ± 0.030 μM (n = 4). In contrast, YM128 and YM-57029 had no effect on PT (data not shown). ADP-induced platelet aggregation was concentration-dependently inhibited by YM-57029, but not by YM128, with an IC₅₀ value of 1.4 ± 0.075 μM (n = 4). In contrast, YM466 had no effect on platelet aggregation (data not shown).

**Ex vivo coagulation and platelet aggregation**
Table 1 summarizes the effects of YM466 and YM128 on PT and platelet aggregation ex vivo. One hour after oral administration, YM466 significantly prolonged PT of the control group by 1.2-fold and 1.5-fold at 30 and 100 mg/kg, respectively. In contrast, YM466 had no effect on platelet aggregation. One hour after oral administration, YM128 significantly inhibited platelet aggregation of the control group by 23 ± 3.7% and 68 ± 5.7% at 10 and 30 mg/kg, respectively. In contrast, YM128 had no effect on PT. Combination of 30 mg/kg YM466 and 10 mg/kg YM128 significantly prolonged PT by 1.2-fold and platelet aggregation by 17 ± 4.7%.

**FeCl₃-induced arterial thrombosis**
Figure 1 shows the antithrombotic effects of YM466 (a), YM128 (b) and a combination of YM466 and YM128 (c) 1 hour after oral administration in a FeCl₃-induced thrombosis model. In the control group, occlusive thrombus was observed in all 14 animals, and time to occlusion ranged from 6.8 to 11.1 minutes (median value: 8.8 minutes). In the YM466 group (Fig. 1A), time to occlusion was prolonged at 10 (P < 0.05) and 30 mg/kg (P < 0.01). Occlusion rate was decreased at 30 mg/kg (P < 0.01). At this dose, no thrombotic occlusion was observed in 5 of the 6 animals within 30 minutes after the initiation of a
vascular injury. In the YM128 group (Fig. 1B), time to occlusion was prolonged at 3 ($P < 0.01$) and 10 mg/kg ($P < 0.01$). Occlusion rate was decreased at 10 mg/kg ($P < 0.01$). At this dose, no thrombotic occlusion was observed within 30 minutes after the initiation of a vascular injury. In the YM466 and YM128 combined group (Fig. 1C), time to occlusion was prolonged at 3 mg/kg YM466 plus 1 mg/kg YM128 ($P < 0.05$) and 10 mg/kg YM466 plus 3 mg/kg YM128 ($P < 0.01$). Occlusion rate was decreased at 10 mg/kg YM466 plus 3 mg/kg YM128 ($P < 0.01$). At this dose, no thrombotic occlusion was observed within 30 minutes after the initiation of a vascular injury.

**Tail transection bleeding time**

Figure 2 shows tail transection bleeding time (BT) 1 hour after oral administration of test drugs. In the control group, bleeding stopped in all 6 animals, and BT ranged from 2.0 to 3.5 minutes (median value: 3.0 minutes). YM466 had no effect on BT up to 30 mg/kg (Fig. 2A). Although YM466 prolonged BT at 100 mg/kg ($P < 0.05$), bleeding was stopped within 30 minutes in all 6 animals. YM128 dose-dependently prolonged BT and bleeding was not stopped within 30 minutes in 3 of the 6 animals and 5 of the 6 animals in the 30 and 100 mg/kg groups, respectively (Fig. 2B). In the combined groups, bleeding time was not prolonged up to 30 mg/kg YM466 plus 10 mg/kg YM128 (Fig. 2C).

**FeCl₃-induced neointima formation**

Figure 3 shows the representative H&E stained histological sections from the normal vessels (Fig. 3A), 2 hours (Fig. 3B) and 3 weeks (Fig. 3C) after vascular injury in the control group and in the combined group of 10 mg/kg YM466 and 3 mg/kg YM128 (Fig. 3F). Figures 3D and 3E show the representative Prussian blue and smooth muscle α-actin stained histological sections 3 weeks after vascular injury in the control group, respectively. Figure 3B revealed the formation of an occlusive, platelet- and fibrin-rich thrombus 2 hours after vascular injury. As shown in Figure 3C, a developed neointima composed of migrated smooth muscle cells was observed 3 weeks after vascular injury, with residual narrow luminal channels. Combination of 10 mg/kg YM466 and 3 mg/kg YM128 strongly inhibited neointima formation 3 weeks after vascular injury, although a small degree of smooth muscle cell proliferation was observed adjacent to the endothelium (Fig. 3F). Prussian blue stain showed that iron uniformly and densely accumulated in the media and adventitia of the blood vessel, but not in the occlusive thrombus in the lumen 2 hours after vascular injury (data not shown). Interestingly, 3 weeks after vascular injury, iron was diffusely observed in the neointima form as well as in the media (Fig. 3D). As shown in Figure 3E, immunohistochemistry confirmed the presence of smooth muscle α-actin-positive cells in neointima (arrows), and these round/globular cells were tentatively identified as proliferating/migrating smooth muscle cells.

Morphometric quantitation of carotid arteries is summarized in table 2 and figure 4. The EEL area, intima area, and intima/media ratio of injured arteries were significantly greater than those of normal arteries (Table 2, $P < 0.01$). However, no significant difference in the lumen and media areas was observed between the two groups. After treatment with YM466 or YM128, the intima area and intima/media ratio were dose-dependently reduced (data not shown). The intima area and intima/media ratio were also dose-dependently reduced by the combination of 3 mg/kg YM466 plus 1 mg/kg YM128 ($P < 0.05$) and 10 mg/kg YM466 plus 3 mg/kg YM128 ($P < 0.01$) (Table 2). In contrast, the EEL area, lumen area and media area were not significantly changed after the use of YM466, YM128 or the combination of the two. The luminal stenosis of the carotid artery was increased 3 weeks after the vascular injury ($P < 0.01$, Fig. 4). The stenosis was inhibited by the administration of 10 mg/kg YM466 ($P < 0.05$), 30 mg/kg YM128 ($P < 0.05$) and the combination of 3 mg/kg YM466 plus 1 mg/kg YM128 ($P < 0.05$).

### Table 1: Prothrombin time and platelet aggregation in mice ex vivo.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Prolongation of prothrombin time (fold of control)</th>
<th>Inhibition of platelet aggregation (inhibition %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM466</td>
<td>10</td>
<td>1.1 ± 0.0097</td>
<td>7.5 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.2 ± 0.034**</td>
<td>11 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.5 ± 0.038**</td>
<td>8.0 ± 4.8</td>
</tr>
<tr>
<td>YM128</td>
<td>3</td>
<td>1.0 ± 0.022</td>
<td>14 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.0 ± 0.020</td>
<td>23 ± 3.7**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.96 ± 0.019</td>
<td>68 ± 5.7**</td>
</tr>
<tr>
<td>YM466 + YM128</td>
<td>3 + 1</td>
<td>0.98 ± 0.015</td>
<td>-8.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>10 + 3</td>
<td>1.1 ± 0.033</td>
<td>8.2 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>30 + 10</td>
<td>1.2 ± 0.016**</td>
<td>17 ± 4.7**</td>
</tr>
</tbody>
</table>

YM466 and YM128 were orally administered 1 hour before the blood collection. Platelet aggregation was induced by 2 μM ADP. Values are mean ± SEM of 6 animals each. *$P < 0.05$ and **$P < 0.01$ by Dunnett’s multiple comparison test compared with the control group.
Figure 1: Antithrombotic effects of YM466 (A), YM128 (B) and a combination of the two (C) on thrombus formation 1 hour after oral administration in a FeCl₃-induced carotid artery thrombosis model of mice. Open circles represent the time to occlusion in each animal. Bars indicate the median values in each group. Numbers of animals are indicated in parentheses. When complete occlusion was not observed within 30 minutes, the time to occlusion was recorded as 30 minutes for statistical analyses. Statistical analyses for time to occlusion and occlusion rate were performed by using Steel’s test and χ²-test, respectively. *: P < 0.05; **: P < 0.01 compared with the control group.

Figure 2: Effects of YM466 (A), YM128 (B) and a combination of the two (C) on tail transection bleeding time 1 hour after oral administration in mice. Open circles represent the bleeding time in each animal. Bars indicate the median values in each group. Numbers of animals are indicated in parentheses. When bleeding was prolonged beyond 30 minutes, the bleeding time was recorded as 30 minutes for statistical analyses. Statistical analyses for bleeding time were performed by using Steel’s test. *: P < 0.05; **: P < 0.01 compared with the control group.
Discussion

In this study, we found that single administration of a factor Xa inhibitor YM466 or a GPIIb/IIIa antagonist YM128 suppressed not only acute thrombosis but also late neointima formation after vascular injury in mice. Furthermore, the combination therapy of YM466 and YM128 produced potent inhibitory effects on thrombosis and neointima formation without affecting coagulation time, platelet aggregation and bleeding time.

In the preliminary studies, the ex vivo anticoagulant activity of YM466 and antiplatelet activity of YM128 reached their peaks at 1 hour after oral administration at 30 mg/kg (unpublished data). Therefore, the ex vivo, and in vivo thrombosis and bleeding studies were performed at 1 hour after oral administration of these drugs. In the in vitro and ex vivo studies, YM466 prolonged PT, without affecting platelet aggregation. In contrast, YM128 and its active metabolite YM-57029 inhibited ADP-induced platelet aggregation, but did not affect PT. Concomitant oral administration of YM466 and YM128 inhibited both coagulation and platelet aggregation ex vivo. This may be attributed to selective inhibition of coagulation and platelet aggregation by each drug, respectively.

Antithrombotic effects of YM466 and YM128 was investigated using a FeCl₃-induced carotid artery thrombosis model in mice (23). In the mechanism of thrombogenesis by FeCl₃, it is postulated that iron ions enhance the conversion of O₂⁻ and...
Table 2: Morphometric parameters of cross-sectional areas of carotid arteries 3 weeks after FeCl₃ injury in mice.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>EEL area (μm²)</th>
<th>Lumen area (μm²)</th>
<th>Intima area (μm²)</th>
<th>Media area (μm²)</th>
<th>Intima/Media ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>6</td>
<td>87042 ± 6859</td>
<td>68736 ± 5732</td>
<td>0 ± 0</td>
<td>18306 ± 1396</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>YM466+YM128</td>
<td>20</td>
<td>130025 ± 6086**</td>
<td>59237 ± 4732</td>
<td>46145 ± 4368**</td>
<td>24643 ± 2120</td>
<td>1.9 ± 0.15**</td>
</tr>
<tr>
<td>1+0.3 (mg/kg)</td>
<td>9</td>
<td>133778 ± 6610</td>
<td>62992 ± 3608</td>
<td>48946 ± 4384</td>
<td>21840 ± 1462</td>
<td>2.3 ± 0.23</td>
</tr>
<tr>
<td>3+1 (mg/kg)</td>
<td>10</td>
<td>127210 ± 6367</td>
<td>72505 ± 6690</td>
<td>30197 ± 3852*</td>
<td>24508 ± 1342</td>
<td>1.2 ± 0.15*</td>
</tr>
<tr>
<td>10+3 (mg/kg)</td>
<td>10</td>
<td>125836 ± 5492</td>
<td>74478 ± 5316</td>
<td>23551 ± 35420**</td>
<td>27806 ± 1883</td>
<td>0.86 ± 0.13**</td>
</tr>
</tbody>
</table>

YM466 and YM128 were orally administered twice daily for 1 week after the surgical operation. Values are mean ± SEM. 

## p < 0.01 by Student’s t-test compared with normal arteries, * p < 0.05 and ** p < 0.01 by Dunnett’s multiple comparison test compared with the control group.

H₂O₂ to oxidizing species, such as hydroxyl radicals which injure endothelial cells (24). An anticoagulant YM466 or an antiplatelet agent YM128 by themselves inhibited the thrombotic occlusion rate at doses that produced the prolongation of PT or the inhibition of platelet aggregation, respectively. These observations were confirmed by our histological analysis which showed that a FeCl₃-induced thrombus mainly consisted of fibrin and platelet (Fig. 3B). In the combination studies, the dose ratio of YM466 to YM128 was 3 to 1 since the antithrombotic effect of YM466 was 3-fold less potent than that of YM128 in this model. As a result, the combination of 10 mg/kg YM466 and 3 mg/kg YM128 completely inhibited the occlusion rate of the injured artery without affecting BT as well as PT and platelet aggregation, although YM466 or YM128 alone requires higher doses to decrease the occlusion rate. These results suggest that the concomitant inhibition of factor Xa and GPIIb/IIIa allow lower the dosage, which leads to a less bleeding risk.

Using the same model as the thrombosis study, we investigated the effects of the antithrombotic agents on neointima formation. Since oxidative stress and lipid peroxidation play major roles in a variety of arterial disorders, such as atherosclerosis (25), hydroxyl radicals produced by FeCl₃ may contribute to neointima formation in this model. Since the anticoagulant activity of YM466 and antiplatelet activity of YM128 after oral administration at 30 mg/kg almost completely disappeared 8 and 12 hours later, respectively (unpublished data), the test drug was administered twice daily in this study. Furthermore, in the preliminary studies, repeated twice-daily administrations of YM466 and YM128 for 3 days showed no change in the peak or duration of their activities (unpublished data). Three weeks after vascular injury, multilayered, α-actin-positive neointima had been generated. Significant increases in the EEL, intima areas, and intima/media ratio, but no significant change in the lumen area or media area, were observed, which is quite consistent with the morphological changes seen in an electrical injury model (26). The lumen area was not significantly changed 3 weeks after vascular injury, despite a significant increase in the intima area. This discrepancy can be explained by the corresponding increase in the size of the vessel, which was evaluated as the cross-sectional area within the EEL. Konstantinides et al. described that iron accumulated at the interface between the thrombus and the vessel wall 30 minutes after injury, but then was rapidly cleared during the wound-healing process (27). However, our histological results showed that iron strongly accumulated in the media and formed neointima 3 weeks after vascular injury. Further studies may be needed to determine whether or not the remaining iron plays a crucial role on late neointima formation in this model. The neointima formation 3 weeks after vascular injury was dose-dependently inhibited by oral administration of YM466 or YM128 twice daily for 1 week. YM466 or YM128 by themselves inhibited luminal stenosis at doses of 10 mg/kg or 30 mg/kg, respectively, suggesting that selective inhibition of factor Xa or GPIIb/IIIa can suppress the development of neointima formation. Furthermore, the combination of lower doses of YM466 and YM128 (3 mg/kg plus 1 mg/kg) significantly suppressed the luminal stenosis without affecting BT, PT and platelet aggregation. Thus, concomitant inhibition of factor Xa and GPIIb/IIIa can suppress neointima formation more effectively and safely than the inhibition of factor Xa or GPIIb/IIIa singly. It should be noted that partial but significant inhibitory effects on neointima formation by YM466 and YM128 were observed in this study. These may have been caused by the twice-daily administration of these agents, irrespective of the relatively short pharmacokinetic properties of these agents as described above. Further studies will be needed to determine whether or not other dose regimens can improve their inhibitory effects. Although neointima formation in the injured artery is the result of a complex process, including coagulation, platelet activation, inflammation, and atherosclerosis, these results suggest the contribution of acute thrombosis to late neointima formation and the complex pathogenesis of luminal stenosis after vascular injury. A relevant question from a clinical point of view is whether or not the single or combined therapy of YM466 and YM128 can reduce cardiac events in patients with ACS. Disappointing findings were obtained with oral GPIIb/IIIa antagonists in large-scale double blind randomized.
trials (17). Although the potential differences between the clinical situation for humans and animal studies may mainly be due to the more complicated heterogeneity of pathophysiology in humans, this question cannot be answered by the present data.

In conclusion, selective inhibition of factor Xa or GPIIb/IIIa effectively suppressed not only acute thrombogenesis but also late neointima formation after vascular injury. Furthermore, the combination therapy of the two may provide a safer and more effective therapeutic regimen for patients with ACS.

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