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Antiaggregatory, antithrombotic effects of MS-180, a novel platelet glycoprotein II_b/III_a receptor antagonist

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Abstract

The antiaggregatory and antithrombotic effects of (*S*)-(−)-ethyl[6-[4-(morpholinoformimidoyl)benzamido]-3,4-dihydro-2*H*-1-benzopyran-3-yl]acetate hydrochloride (MS-180), a novel platelet glycoprotein II_b/III_a receptor antagonist, were investigated. Ma-HCl, (*S*)-(−)-[6-[4-(Morpholinoformimidoyl)benzamido]-3,4-dihydro-2*H*-1-benzopyran-3-yl]acetic acid hydrochloride, the hydrochloride salt of Ma (active metabolite), inhibited the binding of fibrinogen to immobilized human glycoprotein II_b/III_a receptor with an IC_{50} value of 0.12 ± 0.03 nM without affecting binding to either fibronectin or vitronectin receptors. In anesthetized guinea pigs, intraduodenal administration of MS-180 caused dose-dependent inhibition of both ADP- and collagen-induced ex vivo platelet aggregation. At the same dosages, occluded thrombus formation and platelet release reactions were also markedly suppressed. In anesthetized dogs, the bleeding time was prolonged slightly even when submaximal inhibition (< 90%) of ex vivo platelet aggregation was achieved following i.v. administration of Ma-HCl. Aspirin (100 mg/kg) prolonged the bleeding time to the same extent as MS-180 (1 mg/kg), although it suppressed only collagen-induced platelet aggregation. Therefore, MS-180 may be clinically useful for the treatment of thrombotic diseases.

Keywords: MS-180; Glycoprotein II_b/III_a receptor antagonist; Platelet aggregation; Bleeding time; Arterial thrombosis model; Platelet release reaction

1. Introduction

Platelet aggregation and adhesion following platelet activation have been shown to be important processes in occlusive thrombus formation, which is one of major causes of cardiovascular and cerebrovascular diseases such as unstable angina, myocardial infarction, reocclusion after revascularization therapy and stroke (Turner et al., 1995; Coller, 1997; Sandercock, 1997). Aspirin is the most commonly used antiplatelet agent and has been shown to reduce the risk of arterial thrombosis in conditions such as ischemic heart disease and ischemic stroke (Antiplatelet Trialists' Collaboration, 1994; Sandercock, 1997). However, a large proportion of patients do not respond to aspirin therapy (Grotemeyer et al., 1993; Schulman et al., 1996) because of the limited antiaggregatory effect achieved by inhibition of the cyclooxygenase pathway (Patrono, 1994).

Thrombus formation has been demonstrated to be initiated via binding of fibrinogen to the glycoprotein II_b/III_a receptor, which is the final common pathway of platelet aggregation in response to all known agonists (Phillips et al., 1988; Coller, 1995). Antagonism of the glycoprotein II_b/III_a receptor, therefore, is a potentially useful therapeutic target for the treatment of thrombotic diseases. Current clinical investigations with Integrelin (Schulman et al., 1996), abciximab (EPIC Investigators, 1994; Genetta and Mauro, 1996), tirofiban (The Restore Investigators, 1997) or lamifiban (Theroux et al., 1996) as acute intravenous therapies have revealed a favorable efficacy of glycoprotein II_b/III_a receptor antagonists in patients with unstable angina and patients at high risk for acute coronary syndromes, indicating that platelet-mediated thrombus for-

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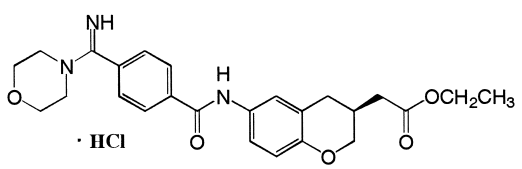
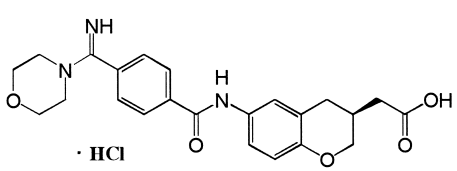
mation contributes significantly to ischemic complications. These observations provided further convincing evidence that orally active glycoprotein II_b/III_a receptor antagonists would have enhanced therapeutic potential in long-term secondary prevention.

Currently, orally active glycoprotein II_b/III_a receptor antagonists are under clinical evaluation (Xemilofiban: Simpfendorfer et al., 1997; Lefradafiban: Muller et al., 1997; Sibrafiban: Christopher et al., 1998). Major concerns in these antiplatelet therapies are the risk of critical bleeding and the efficacy of treatment. Indeed, an increased risk of bleeding complications has been reported in clinical trials with intravenous glycoprotein II_b/III_a receptor antagonists (EPIC Investigators, 1994; Theroux et al., 1996). Therefore, the relationship between bleeding time prolongation and either antiaggregatory efficacy or antithrombotic activity of the oral glycoprotein II_b/III_a receptor antagonists has attracted much attention.

(*S*)-(−)-Ethyl[6-[4-(morpholinoformimidoyl)benzamido]-3,4-dihydro-2*H*-1-benzopyran-3-yl]acetate hydrochloride, MS-180 (Fig. 1), is an ester prodrug and a highly potent antagonist of platelet glycoprotein II_b/III_a receptors (Okumura et al., 1998). Here, we describe the pharmacological profiles of MS-180 and Ma-HCl, the hydrochloride salt of Ma (active metabolite), (*S*)-(−)-[6-4-(Morpholinoformimidoyl)benzamido]-3,4-dihydro-2*H*-1-enzopyran-3-yl]acetic acid hydrochloride. Intraduodenal administration of MS-180 exhibited dose-dependent inhibitory effects on ex vivo platelet aggregation. The relationship between antiaggregatory effects and bleeding time prolongation was determined in both guinea pigs and dogs. In addition to its antiaggregatory effect, MS-180 showed the ability to inhibit platelet release reactions, such as serotonin release, and thromboxane B_2 production. Finally, the antithrombotic efficacy was evaluated in photochemically induced thrombosis models of the guinea pig.



MS-180



Ma-HCl

Fig. 1. Chemical structures of MS-180 and Ma-HCl, the hydrochloride salt of the active metabolite (Ma).

2. Materials and methods

All procedures used in the present study were performed according to the guidelines for animal experiments of the Institute of Biological Science of Mitsui Pharmaceuticals.

2.1. Materials

Both MS-180, (*S*)-(−)-Ethyl[6-[4-(morpholinoformimidoyl)benzamido]-3,4-dihydro-2*H*-1-benzopyran-3-yl] acetate hydrochloride, and Ma-HCl, (*S*)-(−)-[6-[4-(Morpholino-formimidoyl)benzamido]-3,4-dihydro-2*H*-1-benzopyran-3-yl]acetic acid hydrochloride, were synthesized at Mitsui Chemicals, (Chiba Japan). ADP, aspirin and echistatin were purchased from Sigma (St. Louis, MO). Other compounds used were Arg–Gly–Asp–Ser (RGDS; Peptide Institute, Osaka, Japan), Gly–Arg–Gly–Asp–Ser–Pro (GRGDSP; Iwaki Glass, Chiba, Japan), collagen (MC Medical, Tokyo, Japan), vitronectin (Becton Dickinson Labware, MA) and fibronectin (Chemicon International, CA). MS-180 and aspirin were suspended in 0.5% methyl cellulose–0.9% saline and given to fasted guinea pigs for 24 h.

2.2. In vitro receptor binding study

The binding study with human glycoprotein II_b/III_a receptors was performed as described previously (Charo et al., 1991). In brief, the amount of biotinylated fibrinogen that was bound to purified human glycoprotein II_b/III_a complex (Phillips et al., 1992) was measured using an ELISA method in the presence of Ma-HCl or RGDS.

The integrin specificity of Ma-HCl was evaluated by measuring the adhesion of human umbilical vein endothelial cells (Clonetics, CA) to either fibronectin- or vitronectin-coated plates using a modification of the method of Bostwick et al. (1996). Adherent cells in the presence of echistatin, GRGDSP, Ma-HCl or 0.9% saline containing bovine serum albumin (0.1%) were stained with 1% Methylene blue for 1 h and quantified by measuring the absorbance of a 1 N HCl extract (300 μl/well) spectrophotometrically at 620 nm, using an immunoreader (NJ-2000, Inter Med K.K., Tokyo, Japan).

2.3. In vitro platelet aggregation study

For evaluation of the antiaggregatory efficacy of Ma-HCl in different species, ADP- and collagen-induced platelet aggregation in platelet-rich plasma from humans, guinea pigs, dogs, rabbits and rats was determined. Blood samples collected by venipuncture were anticoagulated with 3.8% sodium citrate (9:1 v/v). The extent of platelet aggregation in platelet-rich plasma (2.2×10^5–3×10^5

platelets/μl) was evaluated as the peak response in the 5 min after ADP or collagen stimulation, using an aggregometer (Hematracer PAT-6A, NKK, or Hematracer 212, MC Medical).

2.4. Relationship between ex vivo platelet aggregation and bleeding time

The relationship between the extent of inhibition of ex vivo platelet aggregation and bleeding time prolongation was evaluated in dogs and guinea pigs. In anesthetized dogs, Ma-HCl was given intravenously at sequential doses of 10, 30, 100, 200, 400 and 800 μg/kg at 30-min intervals. Bleeding time was measured 10 min after each dosage and the duration of bleeding time was timed to a maximum of 20 min. In anesthetized guinea pigs, template bleeding time was measured on the skin of the hind limbs using a Simplate R device (Organon Tecknika, Durham, NC). A uniform incision was made and blood was blotted on to filter paper at 30-s intervals until bleeding stopped. For evaluation of the effects of MS-180, intraduodenal administration was selected to eliminate dietary effects throughout the present study. Bleeding time was assessed 1 h after intraduodenal administration of MS-180 or 2 h after p.o. administration of aspirin as described previously (Kawamura et al., 1996). Blood samples were collected for simultaneous measurements of ex vivo platelet aggregation.

2.5. In vivo antithrombotic effect

The antithrombotic effects of MS-180 and aspirin were evaluated in a guinea pig model of thrombosis of the common carotid artery and middle cerebral artery. Transluminal thrombus formation was performed as described previously (Hirata et al., 1993; Umemura et al., 1993). Brief irradiation (10 or 15 min) with green light at 540 nm (L4887, Hamamatsu Photonics, Hamamatsu, Japan) was performed after i.v. administration of rose bengal (5 mg/kg). The artery was considered to be occluded when the blood flow stopped for 1 min or longer, as determined with a pulse Doppler flow probe (PDV-20, Crystal Biotech, Hopkiton, MA). In both models, the irradiation was started 1 h after the intraduodenal administration of MS-180 and 2 h after p.o. administration of aspirin.

2.6. Effect of MS-180 on serotonin release and thromboxane B_2 production during ex vivo platelet aggregation

Thromboxane B_2 production and serotonin release from collagen-stimulated platelets were determined in platelet-rich plasma of guinea pigs receiving either MS-180 or aspirin. After collagen-induced ex vivo platelet aggregation was determined, the plasma was centrifuged (3000 rpm, 10 min, 4°C) with 300 μl of 150 mM saline containing ethylenediaminetetraacetic acid (20 mM) and indomethacin (400 μM). The content of thromboxane B_2 and serotonin in the supernatant was determined simultaneously by enzyme immunoassay (Thromboxane B_2 EIA kit, Cayman Chemical, Ann Arbor, MI; Serotonin EIA kit, Immunotech, Marseille Cedex, France).

2.7. Statistics

Results are expressed as means ± S.D. Between group differences were evaluated by using the Kruskal–Wallis test followed by the non-parametric Dunnett's test (StatLight, Yukms, Tokyo, Japan). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Inhibition of fibrinogen binding by Ma-HCl

Ma-HCl inhibited the binding of biotinylated fibrinogen to immobilized human glycoprotein II_b/III_a complex with an IC_{50} value of 0.12 ± 0.03 nM (results of five independent experiments). RGDS also demonstrated an inhibitory effect on fibrinogen binding with an IC_{50} value of 208 ± 81 nM; the potency was approximately 1700 times weaker than that of Ma-HCl.

3.2. Integrin specificity

Ligand binding to glycoprotein II_b/III_a receptor is primarily mediated via a three-amino acid sequence, Arg–Gly–Asp (RGD). RGD has been shown to mediate cell binding via integrin receptors such as $\alpha_v\beta_3$ (vitronecin receptor) and $\alpha_5\beta_1$ (fibronectin receptor). Therefore, the effects of Ma-HCl, echistatin and GRGDSP on vitronectin ($n = 3$) and fibronectin ($n = 4$) receptors were assessed in the adhesion assay. Ma-HCl had little or no effect on the adhesion of human umbilical vein endothelial cells up to 1 mM. Echistatin caused dose-dependent inhibitory effects

Table 1
Species differences in antiaggregatory efficacy of Ma-HCl against platelet aggregation induced by ADP or collagen

Species	IC_{50} (nM)			
	ADP		Collagen	
Human	32.5 ± 5.6	(4)	58.3 ± 23.5	(4)
Guinea pig	91.9 ± 17.4	(6)	96.8 ± 15.0	(4)
Dog	253.3 ± 133.1	(6)	700.2 ± 76.5	(7)
Rabbit	5000 ± 645	(4)	12800 ± 2910	(4)
Rat	> 1 mM	(5)	> 1 mM	(6)

The concentrations of agonists (ADP in μM, collagen in μg/ml) used were as follows: human (10, 2), guinea pig (5, 2), dog (200, 20), rabbit (50, 20), rat (10, 10).
Values are expressed as means ± S.D. of four to seven experiments indicated in parentheses.

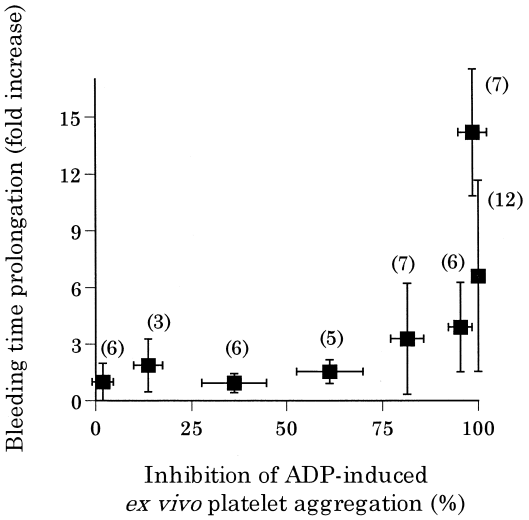


Fig. 2. Relationship between effects of Ma-HCl on ADP-induced ex vivo platelet aggregation and bleeding time. Ex vivo platelet aggregation was elicited with 200 μM ADP. Bleeding time prolongation (fold over basal value) was plotted against extent of inhibition of platelet aggregation of <10%, 11–25%, 26–50%, 51–75%, 76–90%, 91–99% and 100%, and bleeding time of more than 20 min. Basal bleeding time was 94.4±28.5 s ($n=10$). Data are mean±S.D. ($n=3$ to 12 indicated in parentheses).

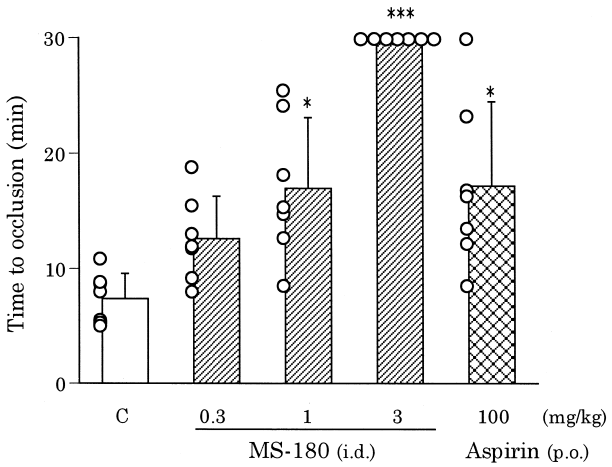


Fig. 3. Effects of MS-180 and aspirin on photochemically induced thrombus formation in the guinea pig common carotid artery. Each column indicates mean time (±S.D.) required for thrombotic occlusion in seven experiments. Open symbols represent values obtained from individual animals. $*P<0.05$, $***P<0.001$ vs. control.

on cell adhesion with IC_{50} values of 0.0093±0.0048 μM for vitronectin receptors and 0.011±0.002 μM for fibronectin receptors. The IC_{50} values of GRGDSP were 64±14 μM for vitronectin receptors and 25±11 μM for fibronectin receptors.

3.3. Effect of Ma-HCl on in vitro platelet aggregation

The antiaggregatory efficacy of Ma-HCl on ADP or collagen-induced platelet aggregation was evaluated in platelet-rich plasma from humans, guinea pigs, dogs, rabbits and rats (Table 1). Ma-HCl produced inhibitory effects on ADP- and collagen-induced platelet aggregation with similar IC_{50} values in both humans and guinea pigs. There were, however, marked species differences in the inhibitory effect of Ma-HCl on platelet aggregation, and the order of inhibitory potency was human > guinea pig > dog ≫ rabbit. Ma-HCl was ineffective in rats.

3.4. Relationship between ex vivo platelet aggregation and bleeding time

Fig. 2 shows the relationship between the extent of inhibition of ADP-induced ex vivo platelet aggregation and bleeding time prolongation during repeated i.v. administration of Ma-HCl in anesthetized dogs. Basal bleeding

Table 2
Effects of MS-180 and aspirin on ex vivo platelet aggregation and bleeding time in guinea pigs

Treatment		Ex vivo platelet aggregation		
		ADP (%)	Collagen (%)	Bleeding Time (s)
Control		87±4	81±3	199±51
MS-180	0.3 mg/kg	48±27	40±33	188±71
	1 mg/kg	20±20[b]	14±28[b]	319±92
	3 mg/kg	−1±5[c]	−4±5[c]	701±226[c]
Aspirin	30 mg/kg	89±4	85±4	233±50
	100 mg/kg	87±6	10±11[a]	341±55[a]

Ex vivo platelet aggregation was elicited with 5 μM ADP and 2 μg/ml collagen.
Values are expressed as mean±S.D. of eight experiments.
[a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ vs. control.

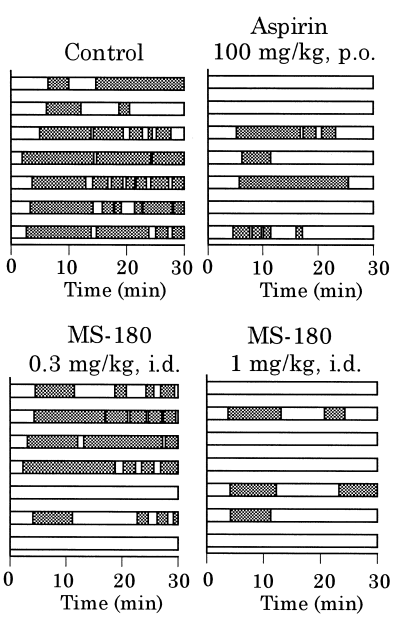


Fig. 4. Schematic representation of the patent status of the middle cerebral artery in individual animals. Irradiation was started at time 0 and was continued for 10 min. Open and dotted portions represent arterial non-occluded (patent) and occluded status, respectively.

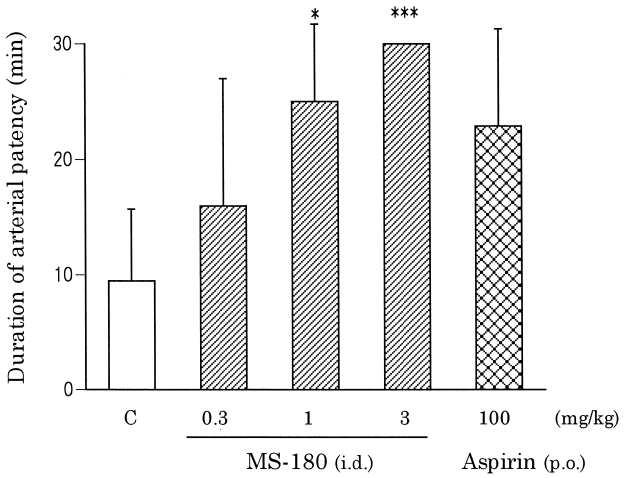


Fig. 5. Effects of MS-180 and aspirin on duration of arterial patency after irradiation in the guinea pig middle cerebral artery. Each column indicates mean ± S.D. ($n = 7$). * $P < 0.05$, *** $P < 0.001$ vs. control.

time was 94.4 ± 28.5 s ($n = 10$). When MS-180 inhibited ADP-induced platelet aggregation by less than 75% compared with baseline values, bleeding time was not markedly altered (120 ± 55.1 s in bleeding time, 1.5 ± 0.6-fold over baseline values at inhibition range of 51% to 75% for ex vivo platelet aggregation, $n = 5$). However, when MS-180 inhibited platelet aggregation by more than 75%, the bleeding time was prolonged in parallel with an increased inhibition of platelet aggregation. In the dog model, however, severe prolongation of the bleeding time was not observed (289.3 ± 240.3 s in bleeding time, 3.3 ± 2.9-fold over basal value, $n = 7$) at dosages where submaximal inhibition of 76 to 90% (81.4 ± 4.4%, $n = 7$) of ex vivo platelet aggregation was achieved following i.v. administration of Ma-HCl.

Further, this relationship was evaluated in guinea pigs receiving either MS-180 or aspirin (Table 2). Intraduodenal administration of MS-180 caused dose-dependent inhibition of both ADP- and collagen-induced ex vivo platelet aggregation. At 1 mg/kg, MS-180 elicited approximately 80% inhibition of platelet aggregation induced by both agonists (ADP: 20 ± 20% vs. 87 ± 4% in control, $P < 0.01$; collagen: 14 ± 28% vs. 81 ± 3% in control, $P < 0.01$) with a slight prolongation of the bleeding time (319 ± 92 s vs. 199 ± 51 s in control, $P > 0.05$). Aspirin at 100 mg/kg p.o. caused a significant inhibition of collagen-induced ex vivo platelet aggregation (10 ± 11% vs. 81 ± 3% in control, $P < 0.05$) but had no effect on ADP-induced ex vivo platelet aggregation (87 ± 6% vs. 87 ± 4% in control, $P > 0.05$). Aspirin (100 mg/kg) prolonged the bleeding time to the same extent as that seen following administration of 1 mg/kg MS-180.

3.5. In vivo antithrombotic effects

The antithrombotic effects of MS-180 and aspirin were determined against photochemically induced thrombosis in the carotid artery and middle cerebral artery of guinea pigs. In both thrombosis models, occlusive thrombi were formed at the site of endothelial injury and arterial occlusion was determined as the cessation of blood flow. In the carotid artery thrombosis model, the carotid flow completely ceased with a mean time to occlusion of 7.4 ± 2.2 min ($n = 7$) after the injection of rose bengal and the occluded status lasted throughout the 30-min observation period in four of the seven animals receiving vehicle alone (Fig. 3). Intraduodenal administration of MS-180 at 1 mg/kg significantly prolonged the time required for arterial occlusion (17.0 ± 6.1 min, $P < 0.05$). Furthermore, in animals receiving 3 mg/kg MS-180, thrombus formation was completely inhibited ($P < 0.001$). Aspirin at a dose of 100 mg/kg p.o. significantly prolonged the time to occlusion (17.2 ± 7.3 min, $P < 0.05$) in this model.

In the middle cerebral artery thrombosis model, the arterial flow ceased with a mean time to occlusion of 4.2 ± 1.8 min ($n = 7$) in the control group. In this model, restoration of blood flow was always observed following arterial occlusion and thereafter patent status alternated with occluded status (Fig. 4). In animals receiving MS-180 at 1 mg/kg i.d., the total time of arterial patency was significantly increased from 9.5 ± 6.2 min to 25.0 ± 6.7 min ($P < 0.05$, Fig. 5), whereas at the same dose the time to occlusion was slightly prolonged (18.9 ± 13.9 min, $P > 0.05$). At 3 mg/kg of MS-180, thrombus formation was completely prevented. Aspirin was less effective against the formation of occlusive thrombus in the middle cerebral artery.

Table 3
Effects of MS-180 and aspirin on thromboxane B_2 production and serotonin release during collagen-induced ex vivo platelet aggregation in guinea pigs

Treatment		Platelet aggregation (%)	Thromboxane B_2 production (ng/ml)	Serotonin release (nM)
Control		77 ± 10	44.73 ± 18.56	230.39 ± 146.40
MS-180	0.3 mg/kg	62 ± 6	30.68 ± 15.98	150.40 ± 165.65
	1 mg/kg	6 ± 6[c]	15.63 ± 7.36[a]	100.47 ± 77.77
	3 mg/kg	1 ± 4[c]	12.55 ± 4.37[b]	43.14 ± 26.96[b]
Aspirin	30 mg/kg	73 ± 18	31.92 ± 13.30	167.52 ± 60.84
	100 mg/kg	21 ± 20[a]	0.69 ± 0.48[c]	70.35 ± 48.72[a]

Ex vivo platelet aggregation was elicited with 2 μg/ml collagen.
Values are expressed as means ± S.D. of seven experiments.
[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ vs. control.

3.6. Effects of MS-180 and aspirin on thromboxane B_2 production and serotonin release during ex vivo platelet aggregation

The effects of intraduodenal administration of MS-180 or p.o. administration of aspirin on thromboxane B_2 production and serotonin release during ex vivo platelet aggregation were studied in guinea pigs. The results are summarized in Table 3. In the control group, thromboxane B_2 production and serotonin release from platelets activated by 2 μg/ml collagen were 44.73 ± 18.56 ng/ml and 230.39 ± 146.40 nM, respectively. Intraduodenal administration of MS-180 prevented the platelet release reaction in parallel with inhibition of collagen-induced ex vivo platelet aggregation. Both thromboxane B_2 production and serotonin release were reduced to 15.63 ± 7.36 ng/ml ($P < 0.05$) and 100.47 ± 77.77 nM ($P > 0.05$) following administration of 1 mg/kg MS-180, a dose which caused approximately 90% inhibition of collagen-induced ex vivo platelet aggregation. Aspirin at 100 mg/kg p.o. significantly inhibited both thromboxane B_2 production (0.69 ± 0.48 ng/ml, $P < 0.001$) and serotonin release (70.35 ± 48.72 nM, $P < 0.05$) from collagen-stimulated platelets.

4. Discussion

We have demonstrated the antiaggregatory and antithrombotic efficacy of MS-180 and Ma-HCl, the hydrochloride salt of Ma, the active metabolite. Ma-HCl inhibited biotinylated fibrinogen binding to purified glycoprotein II_b/III_a complex, resulting in a potent inhibitory effect on platelet aggregation regardless of the agonist used (ADP or collagen). This finding can be explained by the fact that the binding of fibrinogen to the platelet glycoprotein II_b/III_a receptor is the final common pathway of platelet aggregation in response to all known agonists (Coller, 1995). Moreover, Ma-HCl did not affect the adhesion of human umbilical vein endothelial cells to vitronectin or fibronectin receptors, which are members of the RGD-dependent integrin family, as are glycoprotein II_b/III_a receptors. Thus, the high degree of integrin specificity suggests that Ma is a specific antiplatelet compound because glycoprotein II_b/III_a receptor is platelet specific (Coller, 1995), which may be an indication of increased safety. Previous experiments with glycoprotein II_b/III_a receptor antagonists have demonstrated a similar integrin specificity (Aoki et al., 1966; Duggan et al., 1995; Nicholson et al., 1995; Mousa et al., 1996).

As demonstrated with other glycoprotein II_b/III_a receptor antagonists (Kawamura et al., 1996; Cook et al., 1996; Guth et al., 1997), the antiaggregatory activity of Ma-HCl also showed species differences, with sensitivity being the highest for human platelets (Table 1). These observations indicated that guinea pigs and dogs were the preferred species for further assessment of efficacy. Therefore, in the present study either guinea pigs or dogs were used for the evaluation of the ex vivo antiaggregatory effects and the in vivo antithrombotic effects of MS-180 and Ma-HCl.

Clinical trials with intravenous glycoprotein II_b/III_a receptor antagonists such as abciximab (EPIC Investigators, 1994; Genetta and Mauro, 1996) and lamifiban (Theroux et al., 1996) have confirmed significant clinical benefit, but an increased risk of bleeding (6- to 8-fold over basal value) has been observed when platelet aggregation is inhibited by more than 80%. Studies with anesthetized dogs showed that after intravenous administration of Ma-HCl the bleeding time was prolonged as the extent of inhibition of ex vivo platelet aggregation was increased. However, the bleeding time prolongation with Ma-HCl was not critical (3.3-fold over basal value) at doses which led to 76% to 90% inhibition of ADP-induced ex vivo platelet aggregation (Fig. 2). Furthermore, experiments with guinea pigs showed that intraduodenal administration of MS-180 (1 mg/kg) elicited approximately 80% inhibition of ex vivo platelet aggregation with a slight increase in bleeding time (Table 2). Therefore, the present results obtained with guinea pigs and dogs, suggested that significant antiaggregatory activity could be maintained with little risk of severe bleeding time prolongation following administration of MS-180.

In animal experiments with several glycoprotein II_b/III_a receptor antagonists, a dissociation between the effect on bleeding time prolongation and in vivo antithrombotic effect has been documented (Lynch et al., 1995; Kawamura et al., 1997). The in vivo antithrombotic efficacy of MS-180 was determined at doses that did not cause severe prolongation of the bleeding time, using the model of photochemically induced thrombosis of the carotid artery or middle cerebral artery of the guinea pig. The results for both thrombosis models showed that occluded thrombus formation was delayed or substantially prevented following intraduodenal administration of MS-180 at 1 mg/kg, a dose which altered the bleeding time only slightly (Figs. 3 and 5, see Table 2). Takiguchi et al. (1992) clearly demonstrated the involvement of thromboxane A_2, a product of the cyclooxygenase pathway, in thrombus formation at the site of endothelial injury in guinea pigs. Indeed, our study showed significant antithrombotic effects of aspirin at 100 mg/kg (Fig. 3). At this dose, aspirin caused the same degree of inhibition of collagen-induced ex vivo platelet aggregation and bleeding time prolongation as MS-180 (1 mg/kg). Although aspirin is the most commonly used antiplatelet agent (Antiplatelet Trialists' Collaboration, 1994; Sandercock, 1997), a large proportion of patients do not respond to aspirin therapy (Grotemeyer et al., 1993; Schulman et al., 1996) because of the limited antiaggregatory effect achieved by inhibition of cyclooxygenase (Patrono, 1994). Given the antiaggregatory efficacy of Ma regardless of the agonist used, MS-180 would appear to have a greater therapeutic potential than aspirin.

In ex vivo studies with guinea pigs intraduodenal administration of MS-180 dose dependently prevented both thromboxane B_2 production and serotonin release in collagen-stimulated platelets at the same dosages with which significant antiaggregatory and antithrombotic effects were achieved (Table 3, Figs. 3–5). Thromboxane B_2 was measured as a stable thromboxane A_2 metabolite. In animal models, thromboxane A_2 has been shown to cause not only further platelet aggregation but also vasoconstriction (Gresele et al., 1991), accelerating further thrombus formation (Hirata et al., 1993) and reocclusion after thrombolysis therapy (Fitzgerald et al., 1989; Takiguchi et al., 1995). Hirsh et al. (1981) previously demonstrated that patients with unstable angina pectoris have elevated plasma levels of thromboxane A_2 metabolites, which is consistent with the platelet activation seen in the patients. Therefore, it seems that at therapeutic dosages of MS-180, thrombus formation is effectively prevented by the dual inhibition of platelet aggregation and platelet release reaction.

The precise functional role of glycoprotein II_b/III_a receptors in platelet stimulus–response coupling remains controversial. Incubation with monoclonal antibodies against the glycoprotein II_b/III_a receptor led to inhibition of the elevation of intracellular Ca^{2+} concentrations in platelets stimulated with aggregating agents (Powling and Hardisty, 1985). Moreover, glycoprotein II_b/III_a receptor incorporated into phospholipid vesicles has been shown to function as an apparent calcium channel which facilitates calcium influx (Rybak and Renzulli, 1992). Fradafiban, a specific glycoprotein II_b/III_a receptor antagonist, has also been reported to inhibit the platelet release reaction (Carroll et al., 1997). Thus, it has been suggested that the activated glycoprotein II_b/III_a receptor can act as a calcium channel to elevate the intracellular Ca^{2+} concentration and initiate thromboxane A_2 production and serotonin release. However, the functional role of the glycoprotein II_b/III_a receptor remains to be elucidated because not all known glycoprotein II_b/III_a receptor antagonists have the same effect (Foster et al., 1994).

In conclusion, intraduodenally administered MS-180 showed potent antiaggregatory and antithrombotic effects and caused a slight prolongation of the bleeding time in both guinea pigs and dogs. In addition platelet release reaction responses to collagen were prevented at the same dosages that elicited significant antiaggregatory effects. Therefore, MS-180 may be clinically useful for the treatment of thrombotic diseases.

References

Antiplatelet Trialists' Collaboration, 1994. Collaborative overview of randomised trials of antiplatelet therapy: I. Prevention of death, myocardial infarction and stroke by prolonged antiplatelet therapy in various categories of patients. Br. Med. J. 308, 81–106.

Aoki, T., Cox, D., Senzaki, K., Seki, J., Tanaka, A., Takasugi, H., Motoyama, Y., 1966. The anti-platelet and anti-thrombotic effects of FK633, a peptide-mimetic GPIIB/IIIA antagonist. Thromb. Res. 81, 439–450.

Bostwick, J.S., Kasiewski, C.J., Chu, V., Klein, S.I., Sabatino, R.D., Perrone, M.H., Dunwiddie, C.T., Cook, J.J., Leadley, R.J. Jr., 1996. Anti-thrombotic activity of RG13965, a novel platelet fibrinogen receptor antagonist. Thromb. Res. 82, 495–507.

Carroll, R.C., Wang, X.-F., Lanza, F., Steiner, B., Kouns, W.C., 1997. Blocking platelet aggregation inhibits thromboxane A_2 formation by low dose agonists but does not inhibit phosphorylation and activation of cytosolic phospholipase A_2. Thromb. Res. 88, 109–125.

Charo, I.F., Nannizzi, L., Phillips, D.R., Hsu, M.A., Scarborough, R.M., 1991. Inhibition of fibrinogen binding to GP II_b/III_a by a $GPIII_a$ peptide. J. Biol. Chem. 266, 1415–1421.

The TIMI 12 Investigators, Christopher, P.C., MacCabe, C.H., Borzak, S., Henry, T.D., Tischler, M.D., Mueller, H.S., Feldman, R., Palmeri, S.T., Ault, K., Hamilton, S.A., Rothman, J.M., Novotny, W.F., Braunward, E., 1998. Randomized trial of an oral platelet glycoprotein II_b/III_a antagonist, sibrafiban, in patients after an acute coronary syndrome. Results of the TIMI 12 Trial. Circulation 97, 340–349.

Coller, B.S., 1995. Blockade of platelet GP II_b/III_a receptors as an antithrombotic strategy. Circulation 92, 2373–2380.

Coller, B.S., 1997. GP II_b/III_a antagonists: pathophysiologic and therapeutic insights from studies of c7E3 Fab. Thromb. Haemost. 78, 730–735.

Cook, J.J., Holahan, M.A., Lyle, E.A., Ramjit, D.R., Sitko, G.R., Stranieri, M.T., Stupienski, R.F. III, Wallace, A.A., Hand, E.L., Gehret, J.R., Kothstein, T., Drag, M.D., McCormick, G.Y., Perkins, J.J., Ihle, N.C., Duggan, M.E., Hartman, G.D., Gould, R.J., Lynch, J.J. Jr., Lynch, R.J., Manno, P.D., 1996. Nonpeptide glycoprotein II_b/III_a inhibitors: 8. Antiplatelet activity and oral antithrombotic efficacy of L-734,217. J. Pharmacol. Exp. Ther. 278, 62–73.

Duggan, M.E., Naylor-Olsen, A.M., Perkins, J.J., Anderson, P.S., Chang, C.T.-C., Cook, J.J., Gould, R.J., Schaffer, L.W., Smith, R.L., 1995. Nonpeptide fibrinogen receptor antagonists: 7. Design and synthesis of the potent, orally active fibrinogen receptor antagonist L-734,217. J. Med. Chem. 38, 3332–3341.

EPIC Investigators, 1994. Use of a monoclonal antibody directed against the platelet glycoprotein GP II_b/III_a receptor in high risk coronary angioplasty. New Engl. J. Med. 330, 956–961.

Fitzgerald, D.J., Wright, F., Fitzgerald, G.A., 1989. Increased thromboxane biosynthesis during coronary thrombolysis: evidence that platelet activation and thromboxane A_2 modulate the response to tissue-type plasminogen activator in vivo. Circ. Res. 65, 83–94.

Foster, M.R., Hornby, E.J., Brown, S., Hann, M., Kitchin, J., Pike, N., Ward, P., 1994. Inhibition of human platelet aggregation by GR91669, a prototype fibrinogen receptor antagonist. Thromb. Res. 75, 269–284.

Genetta, T.B., Mauro, V.F., 1996. Abciximab: a new antiaggregant used in angioplasty. Ann. Pharmacother. 30, 251–257.

Gresele, P., Deckmyn, H., Nenci, G.G., Vermylen, J., 1991. Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic disorders. TiPS 12, 158–163.

Grotemeyer, K.H., Scharafinski, H.W., Husstedt, I.W., 1993. Two-year follow-up of aspirin responder and aspirin non responder. A pilot-study including 180 post-stroke patients. Thromb. Res. 71, 397–403.

Guth, B.D., Seewaldt-Becker, E., Himmelsbach, F., Weisenberger, H., Muller, T.H., 1997. Antagonism of the GP II_b/III_a receptor with the nonpeptidic molecule BIBU52: inhibition of platelet aggregation in vitro and antithrombotic efficacy in vivo. J. Cardiovasc. Pharmacol. 30, 261–272.

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.

Hirsh, P.D., Hillis, L.D., Campbell, W.B., Firth, B.G., Willerson, J.T.,

1981. Release of prostaglandins and thromboxane into the coronary circulation in patients with ischemic heart disease. New Engl. J. Med. 304, 685–691.

Kawamura, M., Imura, Y., Moriya, N., Kita, S., Fukushi, H., Sugihara, H., Nishikawa, K., Terashita, Z., 1996. Antithrombotic effects of TAK-029, a novel GP II_b/III_a antagonist, in guinea pigs: comparative studies with ticlopidine, clopidogrel, aspirin, prostaglandin E_1 and argatroban. J. Pharmacol. Exp. Ther. 277, 502–510.

Kawamura, M., Tsuji, N., Moriya, N., Terashita, Z., 1997. Effects of TAK-029, a novel GP II_b/III_a antagonist, on arterial thrombosis in guinea-pigs, dogs, and monkeys. Thromb. Res. 86, 275–285.

Lynch, J.J. Jr., Cook, J.J., Sitko, G.R., Holahan, M.A., Ramjit, D.R., Mellott, M.J., Stranieri, M.T., Stabilito, I.I., Zhang, G., Lynch, R.J., Manno, P.D., Chang, C.T.-C., Egbertson, M.S., Halczenko, W., Duggan, M.E., Laswell, W.L., Vassallo, L.M., Shafer, J.A., Anderson, P.S., Friedman, P.A., Hartman, G.D., Gould, R.J., 1995. Nonpeptide glycoprotein II_b/III_a inhibitors: 5. Antithrombotic effects of MK-0383. J. Pharmacol. Exp. Ther. 272, 20–32.

Mousa, S.A., Forsythe, M., Lorelli, W., Bozarth, J., Xue, C.B., Wityak, J., Sielecki, T.M., Olson, R.E., DeGrado, W., Kapil, R., Hussain, M., Wexler, R., Thoolen, M.J., Reilly, T.M., 1996. Novel nonpeptide antiplatelet glycoprotein II_b/III_a receptor antagonist; DMP754: receptor binding affinity and specificity. Coronary Artery Dis. 7, 767–774.

Muller, T.H., Weisenberger, H., Brickl, R., Narjes, H., Himmelsbach, F., Krause, J., 1997. Profound and sustained inhibition of platelet aggregation by Fradafiban, a nonpeptide platelet glycoprotein II_b/III_a antagonist, and its orally active prodrug, Lefradafiban, in men. Circulation 96, 1130–1138.

Nicholson, N.S., Panzer-Knodle, S.G., Salyers, A.K., Taite, B.B., Szalony, J.A., Haas, N.F., King, L.W., Zablocki, J.A., Keller, B.T., Broschat, K., Engleman, V.W., Herin, M., Jacqmin, P., Feigen, L.P., 1995. SC-54684A: an orally active inhibitor of platelet aggregation. Circulation 91, 403–410.

Okumura, K., Shimazaki, T., Aoki, Y., Yamashita, H., Tanaka, E., Banba, S., Yazawa, K., Kibayashi, K., Banno, H., 1998. New platelet fibrinogen receptor glycoprotein IIb–IIIa antagonists: orally active series of a *N*-alkylated amidines with a 6,6-bicyclic template. J. Med. Chem. 41, 4036–4052.

Patrono, C., 1994. Aspirin as an antiplatelet drug. New Engl. J. Med. 330, 1287–1294.

Phillips, D.R., Charo, I.F., Parise, L.V., Fitzgerald, L.A., 1988. The platelet membrane glycoprotein IIb–IIIa complex. Blood 71, 831–843.

Phillips, D.R., Fitzgerald, L., Parise, L., Steiner, B., 1992. Platelet membrane glycoprotein IIb–IIIa complex: purification, characterization, and reconstitution into phospholipid vesicles. In: Hawiger, J.J. (Ed.), Methods in Enzymology, Vol. 215, Platelets: Receptors, Adhesion, Secretion. Academic, San Diego, CA, pp. 244–263.

Powling, M.J., Hardisty, R.M., 1985. Glycoprotein IIb–IIIa complex and Ca^{2+} influx into stimulated platelets. Blood 66, 734–761.

Rybak, M.E.M., Renzulli, L.A., 1992. Effect of calcium channel blockers on platelet GPIIb–IIIa as a calcium channel in liposomes: comparison with effects on the intact platelet. Thromb. Haemost. 67, 131–136.

Sandercock, P., 1997. Antiplatelet therapy with aspirin in acute ischeamic stroke. Thromb. Haemost. 78, 180–182.

Schulman, S.P., Goldschmidt-Clermont, P.J., Topol, E.J., Califf, R.M., Navetta, F.I., Willerson, J.T., Chandra, N.C., Guerci, A.D., Ferguson, J.J., Harrington, R.A., Lincoff, A.M., Yakubov, S.J., Bray, P.F., Bahr, R.D., Wolfe, C.L., Yock, P.G., Anderson, H.V., Nygaard, T.W., Mason, S.J., Effron, M.B., Fatterpacker, A., Raskin, S., Smith, J., Brashears, L., Gottdiener, P., Mee, C., Kitt, M.M., Gerstenblith, G., 1996. Effects of Integrelin, a platelet glycoprotein II_b/III_a receptor antagonist, in unstable angina. A randomized multicenter trial. Circulation 94, 2083–2089.

Simpfendorfer, C., Kottke-Marcharnt, K., Lowrie, M., Anders, R.J., Burns, D.M., Miller, D.P., Cove, C.S., DeFranco, A.C., Ellis, S.G., Moliterno, D.J., Raymond, R.E., Sutton, J.M., Topol, E.J., 1997. First chronic platelet glycoprotein II_b/III_a integrin blockade: a randomized, placebo-controlled pilot study of Xemilofiban in unstable angina with percutaneous coronary interventions. Circulation 96, 76–81.

Takiguchi, Y., Hirata, Y., Wada, K., Nakashima, M., 1992. Arterial thrombosis model with photochemical reaction in guinea-pig and its property. Thromb. Res. 67, 435–445.

Takiguchi, Y., Asai, F., Wada, K., Nakashima, M., 1995. Comparison of antithrombotic effects of $GPII_b/III_a$ receptor antagonist and TXA_2 receptor antagonist in the guinea-pig thrombosis model: possible role of TXA_2 in reocclusion after thrombolysis. Thromb. Haemost. 73, 683–688.

The Restore Investigators, 1997. Effects of platelet glycoprotein II_b/III_a blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty. Circulation 96, 1445–1453.

Theroux, P., Kouz, S., Roy, L., Knudtson, M.L., Diodati, J.G., Marquis, J., Nasmith, J., Fung, A.Y., Boudreault, J., Delage, F., Dupuis, R., Kells, C., Bokslag, M., Steiner, B., Rapold, H.J., on behalf of the investigators, 1996. Platelet membrane receptor glycoprotein II_b/III_a antagonism in unstable angina: the Canadian Lamifiban study. Circulation 94, 899–905.

Turner, N.A., Moake, J.L., Kamat, S.G., Schafer, A.I., Kleiman, N.S., Jordan, R., McIntire, L.V., 1995. Comparative real-time effects on platelet adhesion and aggregation under flowing conditions of in vivo aspirin, heparin, and monoclonal antibody fragment against glycoprotein II_b–III_a. Circulation 91, 1354–1362.

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 rat middle cerebral artery thrombosis model. Stroke 24, 1077–1081.