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European Journal of Pharmacology 366 (1999) 203–213

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Temporary and partial inhibition of platelets by SM-20302 prevents coronary artery thrombosis in a chronic canine model

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Received 17 September 1989; revised 24 November 1998; accepted 4 December 1998

Abstract

We proposed that temporary and partial platelet inhibition by a GPIIb/IIIa receptor antagonist, SM-20302, would provide sustained antithrombotic efficacy in a chronic model of coronary artery thrombosis. Instrumented, conscious dogs received vehicle (Group I, $n = 7$), low dose SM-20302 (30 μg/kg bolus + 1 μg/kg/min infusion for 6 h) (Group II, $n = 7$), or high dose SM-20302 (100 μg/kg bolus + 1 μg/kg/min infusion for 6 h) (Group III, $n = 7$). Thrombosis was initiated by electrolytic injury to the circumflex coronary artery. Coronary blood flow was monitored for 6 h on day 1 and days 2–6. Platelet aggregation was performed in platelet-rich plasma prepared from citrated or heparinized blood. At 6 h, both doses of SM-20302 inhibited adenosine diphosphate-induced platelet aggregation completely (> 90%) in citrated platelet-rich plasma, but incompletely (57–59%) in heparinized platelet-rich plasma. Platelet reactivity returned to baseline values at 24 h. Control animals developed thrombotic occlusion on Day 1. Both doses of SM-20302 maintained vessel patency during the infusion period (Day 1) and the subsequent 5 days. Myocardial infarct size and mortality in the drug treated groups were reduced compared to the vehicle group. Thus, temporary inhibition of platelet reactivity by SM-20302 is associated with sustained prevention of primary thrombus formation, and reduction in infarct size and mortality.

Keywords: Coronary artery thrombosis; GPIIb/IIIa receptor antagonist; Platelet aggregation; Myocardial infarction; SM-20302

1. Introduction

Platelet activation and subsequent aggregation play a pivotal role in the pathophysiology of arterial thromboembolic disorders (Rubenstein et al., 1981; Fitzgerald et al., 1986). Current antiplatelet agents such as aspirin, ticlopidine and clopidgogrel inhibit one of the multiple pathways capable of initiating platelet activation. It is well accepted that the interaction of the platelet GPIIb/IIIa receptor with circulating fibrinogen is an obligatory step in platelet aggregation, irrespective of the mode of activation. Therefore, pharmacological blockade of the interaction between fibrinogen and platelet GPIIb/IIIa receptor has emerged as a therapeutic target in prevention of arterial thrombosis. Chimeric monoclonal antibody (c7E3) to the GPIIb/IIIa receptor was shown to benefit patients undergoing high-risk coronary intervention (EPIC Investigators, 1994a,b). The pharmacokinetic half-life of c7E3 is 7 h (Kleiman et al., 1995) and has a potential problem of immunogenicity (Gold et al., 1990) possibly precluding repeated use. Second generation, low-molecular weight compounds (tirofiban, eptifibatide and lamifiban), which can block the GPIIb/IIIa receptors in a competitive manner, are receiving increasing attention. Thus, tirofiban (RESTORE Investigators, 1997) and eptifibatide (IMPACT-AMI Investigators, 1997) showed promising results in the treatment of acute coronary syndromes. However, the half-life of tirofiban and eptifibatide is 1.6 h (Barrett et al., 1994) and 50–60 min (Charo et al., 1992), respectively, and platelet inhibition is reversed rapidly upon discontinuation of drug. Since current agents pose potential problems of immunogenicity (c7E3) and short survival time in the circulation (eptifibatide, tirofiban), there is a continued effort to develop synthetic GPIIb/IIIa receptor antagonists possessing

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0014-2999/99/$ - see front matter
PII: S0014-2999(98)00912-1

a relatively long-half life, and a favorable platelet inhibition and bleeding time profile.

We investigated the in vivo antithrombotic efficacy of SM-20302, a synthetic, low molecular weight, platelet GPIIb/IIIa receptor antagonist. SM-20302 inhibits the fibrinogen binding to the GPIIb/IIIa receptor without an effect on fibronectin or vitronectin binding (Sakurama et al., 1997). In an initial, acute in vivo study, we demonstrated that SM-20302 produced a dose dependent inhibition of carotid artery thrombus formation secondary to deep arterial wall injury (Rebello et al., 1998a). Primary aim of this investigation was to examine the efficacy of SM-20302 in a chronic canine model of coronary artery injury which culminates in the development of an occlusive thrombus. We hypothesized that limited duration (6 h) of inhibition of the platelet GPIIb/IIIa receptor could lead to long-term (5 days) maintenance of vessel patency despite the presence of a potentially thrombogenic deep arterial wall lesion. One feature of the in vivo evaluation of SM-20302 is that the ex vivo assessment of the platelet inhibition profile was conducted under both normocalcemic and hypocalcemic conditions. This approach stems from previous observations that reduction of plasma ionized calcium by trisodium citrate enhances the observed ex vivo inhibition of aggregation by GPIIb/IIIa receptor antagonists and gives a false indication of the drug's true in vivo antithrombotic efficacy (Phillips et al., 1997; Rebello et al., 1997, 1999). Accordingly, we assessed SM-20302-induced platelet inhibition using blood collected in trisodium citrate (conventional anticoagulant) or heparin with the intention of correlating the in vivo efficacy with the ex vivo platelet aggregation responses.

2. Material and methods

2.1. Materials

SM-20302 was supplied by Sumitomo Pharmaceuticals, (Osaka, Japan). The chemical structure of SM-20302 is shown in Fig. 1. The drug was dissolved in 0.02 N HCl–saline mixture (1–2 ml), and later diluted with saline for intravenous infusion. Trisodium citrate, adenosine diphosphate (ADP), epinephrine, and standard reagents were purchased from Sigma (St. Louis, MO). Heparin Sodium Injection, USP (1000 U/ml) was purchased from Elkins-Sinn, (Cherry Hill, NJ).

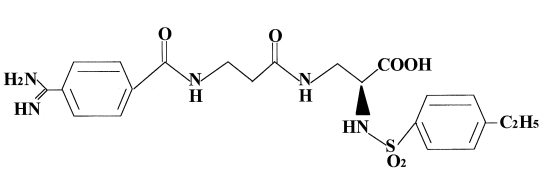

Fig. 1. Chemical structure of SM-20302 (MW = 525.5). 3-[3-(4-caromimidoyl-benzoylamino)-propionylamino]-2-(*S*)-(4-ethylbenzenesulfonylamino)-propionic acid.

2.2. Ethical considerations

Studies conformed to the position of the American Heart Association on research animal use adopted on November 11, 1984. The procedures followed in this study were according to the guidelines of the University of Michigan (Ann Arbor) committee on the use and care of animals. Veterinary care was provided by the University of Michigan unit for laboratory animal medicine. University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards in the guide for care and use of laboratory animals, Department of Health, Education, and Welfare publication no. NIH 78-23.

2.3. Model of coronary artery thrombosis

The animal model used in this study is a modification of one developed by our laboratory for the study of experimentally-induced coronary artery thrombosis. The procedure results in formation of a platelet-rich intravascular thrombus at the site of an electrolytically-induced endothelial lesion in proximity of a distal arterial stenosis (Sudo et al., 1995). In this model, occlusive thrombosis develops in response to vascular injury resulting from application of an anodal current to the intimal surface of the left circumflex coronary artery (Romson et al., 1980; Bates et al., 1992; Lucchesi et al., 1994).

Surgical preparation and instrumentation of the animals has been described in detail previously (Bates et al., 1992; Lucchesi et al., 1994). All surgical procedures were conducted under sterile conditions. Healthy, male or female, purpose-bred beagle dogs (HRP, Kalamazoo, MI) (9–14 kg) were anesthetized with sodium pentobarbital (30 mg/kg, intravenously), intubated, and ventilated with room air and positive pressure at a tidal volume of 30 ml/kg and a frequency of 12 breaths/min (Harvard Apparatus, South Natick, MA). Catheters were inserted into the left carotid artery to monitor the mean arterial blood pressure (Statham P23 pressure transducer, Gould, Oxford, CA) and in the jugular vein to administer vehicle or SM-20302. Catheters were tunnelled under the skin and exteriorized on the dorsal surface of the neck. Heart was exposed by left thoracotomy at the fifth intercostal space and suspended in a pericardial cradle. A 2–3 cm segment of the left circumflex coronary artery was isolated from surrounding tissue by blunt dissection. The left circumflex coronary artery was instrumented with an ultrasonic flow probe (Model 1.5RB24, Transonic Systems, Ithaca, NY). A ligature was formed by tying a suture around an 18 gauge hypodermic needle and the left circumflex coronary artery and then withdrawing the needle. The indwelling coronary

artery electrode consisted of a 30-gauge, Teflon™-insulated, silver-coated copper wire attached to a 25-gauge hypodermic needle tip and inserted through the wall of the left circumflex coronary artery so that the uninsulated needle tip was against the endothelial surface of the vessel. External portion of the stimulating electrode and the flow probe wires were secured to the epicardium by a suture and were exteriorized. The thoracotomy incision was closed and intrathoracic air was evacuated. The surgical wound was dressed, and a nylon jacket (Alice King Chatham Medical Arts, Los Angeles, CA) was placed on the animal to protect the surgical wound site, as well as the externalized flow probe and wire electrode. After recovery from anesthesia, animals were returned to their quarters. In order to avoid the effects of antibiotics on platelet aggregation, antibiotics were not administered intravenously to animals after the surgery (Phillip, 1981).

Animals were returned to the laboratory on the second day and placed in a sling wherein they rested comfortably while conscious and unsedated. The intravascular electrode was connected to the positive pole (anode) of a dual-channel stimulator (Grass S88 stimulator and a Grass constant Current Unit, Model CCU1A, Grass instrument, Quincy, MA) for induction of electrolytic injury. Intensity of the applied current was monitored continuously with an ammeter and maintained at 150 μA. Recordings of mean arterial blood pressure, limb lead II electrocardiogram and coronary artery blood flow were obtained on a model 7 polygraph recorder (Grass Instruments, Quincy, MA). The electrocardiographic recording was examined for evidence of ST-segment alterations coinciding with the fluctuations in coronary artery blood flow.

2.4. Experimental protocol

The protocol for studies designed to determine the efficacy of SM-20302 in the prevention of primary occlusive coronary artery thrombosis is shown in Fig. 2. The experimental protocol was initiated on the day after surgery. Animals were maintained under conscious, unrestrained conditions in a sling, and were randomized to receive either vehicle ($n = 7$), or one of two doses of SM-20302 (30 μg/kg bolus + 1 μg/kg/min infusion for 6 h; $n = 7$, or 100 μg/kg bolus + 1 μg/kg/min infusion for 6 h; $n = 7$). Immediately after vehicle or drug administration, anodal current (150 μA) was applied to the coronary artery for 3 h. Randomization to a specific treatment group was accomplished by blind selection of pre-coded cards. Mean blood pressure, heart rate, coronary artery blood flow and ex vivo platelet aggregation were monitored for 6 h on Day 1. Blood samples were collected from the cephalic vein during infusion for ex vivo platelet aggregation studies and estimating plasma concentration. Dogs were returned to their housing facilities upon completion of the protocol. On each of the subsequent 5 days, surviving dogs were returned to the laboratory. They were allowed to rest in quiet surroundings without the use of anesthesia or sedation, and recordings of coronary artery blood flow were obtained to confirm the presence or absence of flow in the coronary artery and the limb lead II electrocardiogram was monitored for electrocardiographic changes suggestive of ischemic injury. The artery was defined as patent when the measured blood flow was > 1 ml/min for > 10 min. Venous blood samples were withdrawn on the second day for cell counts and aggregation studies. The dogs were returned to the holding rooms each day after completion of the daily recordings and data acquisition.

All surviving dogs were euthanized on Day 6 by an overdose of sodium pentobarbital. After spontaneous death or euthanasia, the chest was opened and the heart was removed. Chest and abdominal cavity was examined for any signs of internal hemorrhage. The coronary artery was dissected free as far as possible and opened longitudinally. Intracoronary position of the electrode was verified and the thrombus was removed and weighed. Heart was sectioned

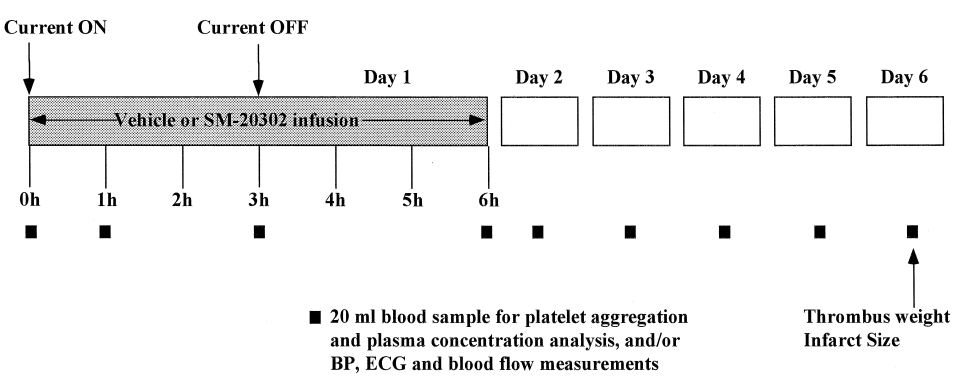

Fig. 2. Diagrammatic representation of the experimental protocol to investigate the in vivo efficacy of SM-20302 in a chronic canine model of coronary artery thrombosis. Surgically instrumented conscious dogs were randomized to receive either vehicle ($n = 7$), or one of two doses of SM-20302 (30 μg/kg bolus + 1 μg/kg/min infusion for 6 h; $n = 7$, or 100 μg/kg bolus + 1 μg/kg/min infusion for 6 h; $n = 7$), following which electrolytic injury was initiated. Hemodynamic parameters and blood flow were monitored throughout the protocol. Periodic venous blood samples were drawn in citrate and heparin to assess the platelet aggregation.

from apex to base in 1.0 cm-thick sections that were incubated, without agitation, in triphenyltetrazolium chloride for 5 min at 37°C. The transverse ventricular sections were weighed and traced onto clear acetate sheets. The red pigmented tissue containing the precipitated formazan complex represented viable tissue, whereas regions that remained pallid indicated infarcted tissue. Demarcated areas were scanned on a flatbed scanner and digitized using a Macintosh IIci microcomputer (Apple Computer, Cupertino, Innovative Data Design, Concord, CA). Infarct size was expressed as a percentage of the total ventricular mass.

2.5. Hematologic measurements

Blood (10 ml) was withdrawn into a plastic syringe containing 3.7% sodium citrate as the anticoagulant [1:10 citrate/blood (v/v)]. An additional 10 ml of whole blood was collected in heparin (10 U/ml blood). The platelet count was determined with an H-10 cell counter (Texas International Laboratories, Houston, TX). Platelet-rich plasma, the supernatant present after centrifugation of anticoagulated whole blood at 1000 rpm (140 g) for 5 min, was diluted with platelet-poor plasma to achieve a platelet count of 200,000/μl. Platelet-poor plasma was prepared after the platelet-rich plasma was removed by centrifuging the remaining blood at 5000 rpm (2000 g) for 10 min and discarding the bottom cellular layer. Ex vivo platelet aggregation was assessed by established nephelometric methods with a four-channel aggregometer (BioData-PAP-4, BioData, Hatboro, PA) by recording the increase in light transmission through a stirred suspension of platelet-rich plasma maintained at 37°C. Aggregation was induced with ADP (20 μM). A subaggregatory dose of epinephrine (550 nM) was used to prime the platelets before stimulation. Values for platelet aggregation are expressed as percentage of light transmission standardized to platelet-rich plasma and platelet-poor plasma samples yielding 0% and 100% light transmission, respectively.

2.6. Estimation of SM-20302 plasma concentration by high performance liquid chromatography (HPLC) analysis

SM-20302 was analyzed by HPLC after extraction from citrated plasma samples. Plasma (1 ml) was mixed with 50 μl of SL-10579 (10 μg/ml; internal standard for SM-20302) solution and 3 ml of sodium acetate buffer (10 mM; pH 5). The mixture was applied to a Bond Elut certify-II column, which was preconditioned with methanol, followed by 10 mM sodium acetate buffer. The column was rinsed with 3 ml of 10 mM sodium acetate buffer and 3 ml of 10% aqueous methanol solution. The eluate was evaporated at room temperature and dissolved in 0.2 ml of the mobile phase (0.1% trifluoroacetic acid in 22% of aqueous acetonitrile solution).

Chromatography was performed using a Puresil C18 column (4.6 mm i.d. × 25 cm, 5 μm), flow rate of 1 ml/min, sample injection volume of 50 μl and column temperature of 35°C. The eluate was monitored at 230 nm by a UV detector (Shimadzu SPD-10AD, Kyoto, Japan). Plasma concentration of SM-20302 was calculated from a standard curve prepared by spiking known quantities of the drug in blank plasma samples.

2.7. Inclusion criteria

Purpose-bred animals included in the final protocol satisfied the following preestablished criteria: (1) a circulating platelet count of not less than 100,000/μl; (2) demonstrated ability for epinephrine-primed platelets to aggregate in response to ADP before administration of vehicle or SM-20302; and (3) absence of heart worms at final postmortem examination. All animals initially selected met the inclusion criteria.

2.8. Statistical analysis

The data are represented as mean ± S.E.M. A one-way analysis of variance (ANOVA) (repeated measures) was used to assess differences in blood flow, hemodynamics and whole blood cell counts over time within groups I, II and III. One-way analysis of variance (factorial) was used for group comparisons. Fisher's protected least significant difference (PLSD) and Bonferroni/Dunn post hoc analysis were used to determine significance at $P < 0.05$. A paired *t*-test was employed to assess the differences in platelet aggregation over time within a group, and values were determined to be statistically different at a level of $P < 0.05$. The incidence of occlusion and mortality between groups was compared using Fisher's Exact Test.

3. Results

3.1. Group characteristics

A total of 24 dogs, which met the preset criteria, were entered into the study. However, three dogs were excluded during the protocol. One dog succumbed to ventricular fibrillation during surgery, and two dogs had a malfunction of flow probe on Day 1 of the protocol. Therefore, 21 dogs were randomized to the following three experimental groups:

Group I. Control: vehicle infusion (8 ml/h for 6 h) ($n = 7$)

Group II. Low dose SM-20302 (30 μg/kg bolus + 1 μg/kg/min infusion for 6 h) ($n = 7$)

Group III. High dose SM-20302 (100 μg/kg bolus + 1 μg/kg/min infusion for 6 h) ($n = 7$)

There was no significant difference among the groups in terms of the body weight (12.16 ± 0.57 kg, 11.1 ± 0.85 kg and 11.94 ± 0.71 kg).

Table 1
Systemic hemodynamics

Time	Blood pressure (mm Hg)			Heart rate (beats/min)		
	Group I	Group II	Group III	Group I	Group II	Group III
Baseline	111 ± 6	106 ± 5	96 ± 11	136 ± 11	149 ± 16	110 ± 17
1 h	101 ± 8	103 ± 5	103 ± 10	129 ± 11	154 ± 19	106 ± 21
3 h	107 ± 6	103 ± 8	96 ± 5	148 ± 15	133 ± 16	107 ± 21
6 h	93 ± 8	101 ± 5	96 ± 9	147 ± 10	130 ± 17	106 ± 16
Day 2	86 ± 7	100 ± 4	103 ± 17	165 ± 15	132 ± 10	140 ± 24
Day 3	86 ± 6	98 ± 5	89 ± 16	143 ± 19	118 ± 11	130 ± 18
Day 4	106 ± 18	93 ± 5	92 ± 22	143 ± 32	121 ± 9	114 ± 17
Day 5	82 ± 6	86 ± 4	80 ± 16	129 ± 9	129 ± 14	107 ± 14
Day 6	100 ± 12	85 ± 4	81 ± 16	140 ± 10	121 ± 12	110 ± 19

Group I = saline infusion (8 ml/h) ($n = 7$).
Group II = 30 μg/kg + 1 μg/kg/min, 6 h ($n = 7$).
Group III = 100 μg/kg + 1 μg/kg/min, 6 h ($n = 7$).

3.2. Hemodynamic parameters

Blood pressure and heart rate were not affected by any of the dosing regimens listed above (Table 1). In the vehicle-treated animals, the lead II electrocardiogram (EKG) exhibited abnormal Q-waves and ST-segment depression on Day 6, indicative of myocardial infarction. Dogs treated with SM-20302 essentially had normal EKGs, and showed little or no evidence of ST-segment changes.

3.3. Plasma concentration of SM-20302

Parallel measurements of SM-20302 concentration in plasma were performed during the protocol. Infusion of 30 μg/kg bolus plus an infusion of 1 μg/kg/min resulted in plasma concentrations of 53 ± 10, 53 ± 6 and 58 ± 7 ng/ml at 1, 3 and 6 h after initiating drug administration. Similarly, the 100 μg/kg bolus plus an infusion of 1 μg/kg/min resulted in plasma concentrations of 49 ± 8, 52 ± 18 and 44 ± 13 ng/ml at 1, 3 and 6 h after infusion. There was no statistically significant difference between the plasma concentrations in Groups II and III ($P = 0.86$). Drug was not detected in plasma collected on Days 2–6.

3.4. Circulating blood cell counts

Effects of SM-20302 on whole blood cell counts, hemoglobin and hematocrit are shown in Table 2. One way ANOVA analysis (repeated measures) indicated that all the groups had similar hematocrit ($P = 0.11$) and platelet counts ($P = 0.35$). Circulating cell counts during the first

Table 2
Whole blood cell counts

		Baseline	1 h	3 h	6 h	Day 2
RBC ($\times 10^3/\mu l$)	Group I	9 ± 0.2	9 ± 0.1	9 ± 0.1	9 ± 0.1	8 ± 0.1
	Group II	9 ± 0.6	9 ± 0.9	8 ± 0.3	8 ± 0.5	8 ± 0.1
	Group III	9 ± 0.6	9 ± 1	8 ± 0.5	8 ± 1	8 ± 0.3
WBC ($\times 10^3/\mu l$)	Group I	25 ± 3	23 ± 5	28 ± 3	46 ± 18	21 ± 7
	Group II	24 ± 4	20 ± 1	21 ± 2	18 ± 2	16 ± 1
	Group III	25 ± 6	23 ± 7	33 ± 7	23 ± 7	18 ± 7
Hb (%)	Group I	41 ± 7	41 ± 6	43 ± 9	36 ± 2	35 ± 1
	Group II	35 ± 0.5	36 ± 1	36 ± 0.8	43 ± 7	39 ± 4
	Group III	40 ± 13	35 ± 1	35 ± 1	35 ± 0.5	41 ± 15
HCT (%)	Group I	51 ± 2	51 ± 2	51 ± 3	49 ± 3	47 ± 3
	Group II	52 ± 2	49 ± 1	48 ± 2	46 ± 1	47 ± 3
	Group III	48 ± 5	42 ± 5	41 ± 4	42 ± 8	42 ± 8
PLT ($\times 10^3/\mu l$)	Group I	551 ± 55	511 ± 57	489 ± 37	471 ± 57	435 ± 39
	Group II	628 ± 56	554 ± 34	540 ± 40	534 ± 32	460 ± 59
	Group III	555 ± 130	473 ± 136	516 ± 109	524 ± 183	405 ± 76

Group I = saline infusion (8 ml/h) ($n = 7$).
Group II = 30 μg/kg + 1 μg/kg/min, 6 h ($n = 7$).
Group III = 100 μg/kg + 1 μg/kg/min, 6 h ($n = 7$).

6 h and again at 24 h after drug administration did not reveal significant changes.

3.5. Platelet aggregation studies

Ex vivo platelet aggregation in response to ADP was determined at $T = 0$ (predrug), 1, 3 and 6 h during SM-20302 infusion, and also on Day 2. Platelet aggregations were performed in citrated platelet-rich plasma and heparinized platelet-rich plasma. At baseline, the percent platelet aggregation in response to ADP in citrated platelet-rich plasma were 64%, 63% and 68% for Groups I, II and III, respectively (Fig. 3). Similarly, the percent platelet aggregation in response to ADP at baseline in heparinized platelet-rich plasma were 74%, 86% and 71% for Groups I, II and III, respectively (Fig. 4). The baseline platelet aggregations in citrated platelet-rich plasma and heparinized platelet-rich plasma were statistically similar in all groups.

The 6-h infusion of SM-20302 (II and III) produced complete inhibition (> 90%) of ADP-induced platelet aggregation in citrated platelet-rich plasma during the infusion period (Fig. 3). In heparinized platelet-rich plasma, however, a partial inhibition was observed (Fig. 4). The maximum inhibition observed in Groups II and III was 59% and 57%, respectively. There was no significant difference in the extent of platelet inhibition between the two infusion regimens. At 24 h (Day 2), the platelet aggregation returned to baseline (pre-drug) values, indicating reversible action of SM-20302.

3.6. Coronary artery blood flow

Coronary blood flow was monitored daily with the use of a chronically implanted ultrasonic flow probe on the left circumflex coronary artery. The data are presented graphically in Fig. 5. The baseline left circumflex coronary artery coronary artery flow values for Groups I, II and III were 11 ± 2, 11 ± 2 and 10 ± 2 ml/min, respectively ($P > 0.05$). In the vehicle-treated animals (Group I), electrolytic injury to the endothelial surface of the coronary artery produced cyclic variations in the flow pattern culminating in complete occlusion (mean time to occlusion = 110 ± 25 min). Occlusion of the artery was accompanied by characteristic changes in the lead II electrocardiogram showing initial elevation and then persistent depression in the ST segment and finally development of Q-waves. The left circumflex coronary artery coronary artery remained occluded for the remainder of the protocol (Days 2–6).

Infusion of SM-20302 was associated with a significant ($P = 0.007$) maintenance of vessel patency as compared to the control group. Despite the presence of a deep vessel

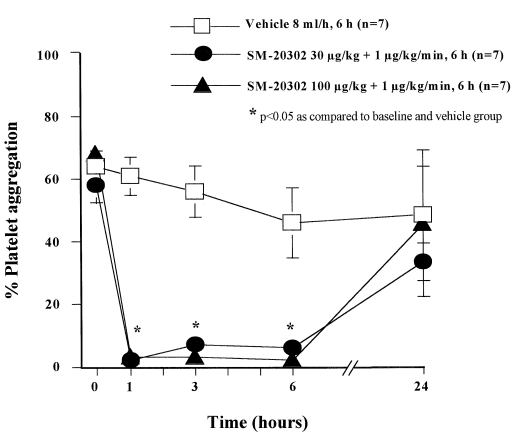

Fig. 3. Effect of a 6-h infusion of SM-20302 or vehicle on ex vivo platelet aggregation induced by ADP (20 μM) in citrated platelet-rich plasma. Values are expressed as mean ± S.E.M. of the percent platelet aggregation values. Preselected time intervals represent time during the coronary artery thrombosis protocol.

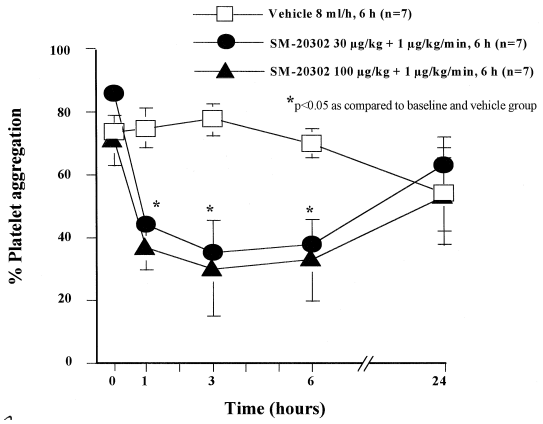

Fig. 4. Effect of a 6-h infusion of SM-20302 or vehicle on ex vivo platelet aggregation induced by ADP (20 μM) in heparinized platelet-rich plasma. Values are expressed as mean ± S.E.M. of the percent platelet aggregation values. Preselected time intervals represent time during the coronary artery thrombosis protocol.

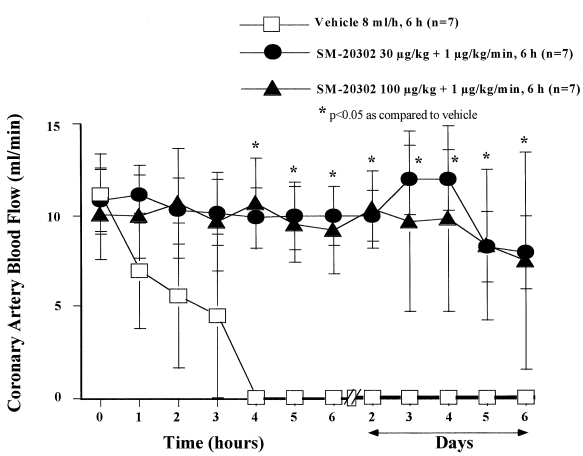

Fig. 5. Coronary artery blood flow during the thrombosis protocol. Electrolytic injury produced complete and persistent occlusion of the coronary artery in all the vehicle-treated dogs. Infusion of SM-20302 not only maintained coronary artery patency for the duration of infusion but also for five additional days. Values are expressed as mean ± S.E.M.

Table 3
Occlusion parameters and mortality

Groups	Incidence of occlusion	Time to occlusion	Thrombus weight (mg)	Infarct size (% of LV)	Mortality
Group I	7/7	110.4 ± 25.2 min	7.7 ± 1.4	28.7 ± 4.7	4/7 (57%)
Group II	2/7[a]	3.5 ± 1.5 days	3.8 ± 0.4[a]	3.3 ± 0.9[a]	1/7 (14%)
Group III	2/7[a]	4.5 ± 1.5 days	1.8 ± 1.0[b]	4.8 ± 7.5[a]	0/7 (0%)[a]

[a] $P < 0.05$.
[b] $P < 0.01$ compared to Group I.
Group I = saline infusion (8 ml/h) ($n = 7$).
Group II = 30 μg/kg + 1 μg/kg/min, 6 h ($n = 7$).
Group III = 100 μg/kg + 1 μg/kg/min, 6 h ($n = 7$).

wall injury, the coronary artery blood flow in groups II and III at the end of constant infusion ($T = 6$ h) was 93% ($P = 0.06$) and 92% ($P = 0.10$) of the baseline value. Furthermore, both infusion regimens of SM-20302 prevented subsequent reduction in the coronary artery blood flow on Days 2–6. At the end of the protocol (Day 6), the blood flow was 73% ($P = 0.46$) and 75% ($P = 0.17$) of the baseline for groups II and III, respectively. The lead II EKG in these dogs was normal in morphology and did not show signs of ischemia, infarction or arrhythmia.

The incidence of occlusion over the six days of the experimental protocol is summarized in Table 3. Whereas 7/7 (100%) of the vehicle-treated animals had occluded vessels, the SM-20302-treated groups had only 2/7 occluded vessels (29%, Groups II and III) ($P < 0.05$). Moreover, the time to occlusion for the two animals in each of the SM-20302-treated groups was delayed significantly.

3.7. Thrombus weight

Thrombi were removed either after spontaneous death of the animal or at the time of euthanasia on Day 6. Macroscopic examination of the injured segment revealed precise electrode placement and presence of deep arterial wall injury in all animals. Thrombus weight was reduced significantly in the group of animals treated with SM-20302 when compared with the thrombi removed from the control animals (Table 3).

3.8. Mortality

Two of the vehicle-treated animals succumbed to sudden death due to ventricular fibrillation, immediately after occlusion of the coronary artery. By Day 3, two additional vehicle-treated animals, although active and in no apparent discomfort, died suddenly. While not documented electrocardiographically, it is assumed that death was due to an arrhythmic event. The mortality rates in the SM-20302-treated Group II (14%; $P = 0.12$) and Group III (0%; $P = 0.03$) were lower as compared to the controls (57%) (Table 3). Therefore, SM-20302 administration was associated with an increased survival.

3.9. Myocardial infarct size

In the control group, one animal developed ventricular fibrillation, but showed no evidence of infarction due to the brief period of ischemia. The mean infarct size, expressed as a percent of the left ventricle, in the remaining six animals was 28.7 ± 4.7% (Table 3). In the SM-20302-treated animals, there was minimal evidence of myocardial infarction as assessed by the tetrazolium method. The infarcted regions for Groups II (3%) and III (5%) were significantly ($P < 0.05$) smaller when compared with that of the control group.

4. Discussion

We investigated the pharmacological profile of SM-20302, a synthetic GPIIb/IIIa receptor antagonist using a chronic canine model of coronary artery thrombosis. The model employed (Romson et al., 1980) was designed specifically for examining the therapeutic potential of antithrombotic agents. The experimental model utilizes electrolytic injury to the intimal surface of the artery to form a thrombus that is mixed (platelets, red blood cells and white blood cells) in composition (Sudo et al., 1995). The model has been validated successfully using such GPIIb/IIIa receptor antagonists as 7E3 (Mickelson et al., 1989), SC-49992 (Rote et al., 1993) and DMP-728 (Lucchesi et al., 1994). Employing SM-20302, a GPIIb/IIIa receptor antagonist, we found that temporary (< 24 h) and partial (57–59%) inhibition of platelet reactivity was associated with a long-term (5 days) prevention of arterial thrombosis, a reduction in myocardial infarct size and mortality in conscious dogs. SM-20302 was well tolerated and we did not observe any obvious signs of bleeding episodes from the surgical access or venipuncture sites.

An important feature of the present study is that we examined the ex vivo platelet inhibition profile under hypocalcemic (trisodium citrate) and normocalcemic (heparin) conditions. The main reason for this approach derives from the recognition (Phillips et al., 1997; Schneider et al., 1997) that the use of the conventional anticoagulant, trisodium citrate, introduces a non-physiologic

environment (removal of ionized calcium) in the ex vivo evaluation of antiplatelet agents. Extracellular calcium ions stabilize the GPIIb (Heidenreich et al., 1990; Kieffer and Phillips, 1990) and GPIIIa (Loftus et al., 1990; Lee et al., 1995; Tozer et al., 1996) subunits of the integrin receptor. Divalent cations such as Ca^{2+} and Mn^{2+} can compete with Arg–Gly–Asp-containing ligands or synthetic antagonists for binding to the activated GPIIb/IIIa receptor (D'souza et al., 1994; Hu et al., 1996). Trisodium citrate chelates the extracellular ionized calcium that is essential for the formation of the GPIIb/IIIa heterodimer (Lam, 1992), and for the interaction between the GPIIb/IIIa receptor and fibrinogen (Steiner et al., 1989). Under conditions in which ionized calcium is depleted or reduced, platelets are less capable of binding to fibrinogen in response to ADP (Marguerie et al., 1979; Peerschke et al., 1980; Lanza et al., 1992). Due to the decreased ionized calcium concentration in the citrated platelet-rich plasma, the binding of GPIIb/IIIa receptor antagonist and subsequent inhibition of platelet reactivity is enhanced as demonstrated for integrilin (Phillips et al., 1997). This leads to an overestimation of the antagonistic potency that deviates significantly from that observed in the in vivo environment where the ionized calcium concentration is maintained within the physiologic range, thereby allowing the antagonist to interact with the GPIIb/IIIa receptor under normocalcemic conditions. For example, we have found that the in vitro IC_{50} of c7E3, MK-383, DMP-728 and SM-20302 for inhibiting ADP or thrombin receptor activating peptide-induced human platelet aggregation in citrated platelet-rich plasma was ~2-fold lower than that in platelet-rich plasma prepared from blood anticoagulated with D-phenylalanyl-L-propyl-L-arginyl-chloromethylketone (Rebello et al., 1999). The dichotomy in the platelet inhibition profile in citrated platelet-rich plasma vs. heparinized platelet-rich plasma was confirmed in the present study.

Introduction of GPIIb/IIIa receptor antagonists as a new class of therapeutic agents raises an important question of drug monitoring and establishing a therapeutic dosing regimen. To date, clinical experience with c7E3 has suggested that an >80% receptor blockade is required for maintaining antithrombotic efficacy (Coller, 1997). Although assessing the extent of receptor blockade may provide accurate information about antithrombotic efficacy, it is cumbersome to adapt the method for a point-of-care use due to the need of extensive instrumentation and technical expertise. On the other hand, the ex vivo examination of platelet reactivity performed in platelet-rich plasma prepared from citrate anticoagulated blood, may yield misleading information about the in vivo efficacy of GPIIb/IIIa receptor antagonists (Phillips et al., 1997; Schneider et al., 1997; Rebello et al., 1999). The citrate-induced distortion in the plasma ionized calcium concentration leads to a discrepancy when comparing the ex vivo and in vivo efficacy of platelet GPIIb/IIIa receptor antagonists. It was evident from our earlier studies with SM-20302 (Rebello et al., 1998a) and TP-9201 (Rebello et al., 1997) that a partial (40–60%) inhibition of ex vivo platelet aggregation in heparinized platelet-rich plasma was required to exhibit in vivo antithrombotic efficacy. Furthermore, a dose-dependent inhibition of ADP-induced platelet aggregation in heparinized platelet-rich plasma by SM-20302 correlated with the graded prolongation in bleeding time (Rebello et al., 1998a). Therefore, knowing the pharmacokinetic parameters of SM-20302 (plasma clearance = 7.54 ml/min/kg and terminal half-life = 188 min) (Rebello et al., 1998b), we designed a bolus plus infusion regimen with the aim of maintaining a concentration that would approximate the IC_{50} value (~79 ng/ml) of SM-20302 necessary for inhibiting ADP-induced platelet aggregation in heparinized platelet-rich plasma. No information is published in the literature regarding the window of thrombogenicity in the electrolytic injury model to assist to target drug therapy during that window. Therefore, we used two different loading doses in order to find the lowest effective dose and to maximize the safety profile. Since we first observed efficacy with the 100 μg/kg + 1 μg/kg/min infusion, we thought perhaps the infusion regimen is more important, and that a lower bolus with the same infusion regimen may be equally effective. The results demonstrated that both dosing regimens were equally effective in partially (57–59%) inhibiting the platelet aggregation response and were identical with respect to compound exposure. For these reasons no conclusion may be drawn about the dose dependency of any of the observed effects. Because the two groups differed only in bolus doses it is likely that the time to steady state and plasma concentrations were different initially (before the 1 h time point), but the results suggest that this did not compromise the efficacy. The data with SM-20302 suggest that one could effectively monitor the therapy with a platelet GPIIb/IIIa receptor antagonist, and predict efficacy and/or safety by performing ex vivo platelet inhibition assays under conditions in which the platelet-rich plasma contains a physiologic concentration of ionized calcium.

It is important to note that although the inhibition of platelet aggregation was reversed after cessation of the infusion, the vessel patency was maintained for five additional days despite the presence of a deep arterial wall injury. As a result, the SM-20302-treated dogs had a decreased thrombus mass in the injured coronary artery and, in particular, infarct size was reduced significantly when compared to the vehicle-treated dogs. Therefore, SM-20302-induced short-term inhibition of platelet reactivity provided sufficient time for resolution of the thrombogenic reactivity of the injured vessel wall. The poorly understood process of 'passivation' abrogates the platelet-vessel wall interaction despite the continued presence of a deep vessel wall injury. Our laboratory previously documented the phenomenon of 'passivation' using two other

structurally different GPIIb/IIIa receptor antagonists, 7E3 (Bates et al., 1992; Rote et al., 1994) and DMP-728 (Lucchesi et al., 1994). In these studies it was noted that temporary, but complete inhibition of platelet aggregation was associated with maintenance of vessel patency for 5 days. A complete inhibition of platelet aggregation was observed in the latter studies because the ex vivo platelet aggregation determinations were performed in citrated platelet-rich plasma. Collectively, the results with SM-20302, 7E3 and DMP-728 provide evidence that in order to achieve 'passivation' one must inhibit platelet reactivity during the period wherein the injured vessel is highly thrombogenic. In the electrolytic injury model of thrombosis, the window of thrombogenicity appears to be the first 6 h after induction of the deep vascular lesion that results in exposure of subendothelial structures (Romson et al., 1980; Bates et al., 1992). The precise mechanism for the SM-20302-induced 'passivation' is not clear. It may be postulated that the 6-h infusion of the antagonist may have prevented platelet aggregation during the 3-h period of intense arterial injury, thereby allowing the disrupted surface of the coronary artery to become non-thrombogenic through adsorption of plasma proteins at the site vessel wall injury. Preadsorption of a thrombogenic surface with albumin is known to reduce platelet deposition and thrombus formation (Mulvihill et al., 1990). Treatment of rabbits with prostacyclin, or dipyridamole for 8 h prevented the deposition of platelets on the aortas during angioplasty when the latter was performed in the absence of drugs (Groves et al., 1986). Groves et al. (1979) quantified the accumulation of ^{51}Cr-labeled platelets on the subendothelium of rabbit aortas after injury with a balloon catheter. By injecting ^{51}Cr-labeled platelets into rabbits at different times, it was found that a monolayer of platelets formed initially on the injured surface, and the number of platelets associated with the surface decreased over a 7-day period. Mascelli et al. (1998) examined the pharmacodynamic profile of short-term abciximab (c7E3, ReoPro™) treatment which was accompanied by prolonged platelet inhibition due to the continuous re-equilibration of the antibody among circulating platelets for a period of 15 days. Partial restoration of platelet function and a decrease in GPIIb/IIIa receptor blockade ($< 80\%$) was observed at 12 h post drug. Platelet function was restored to normal within 24–36 h despite the continued presence of c7E3 antibody bound to the circulating platelets, albeit in a concentration that blocked less than 80% of the available receptors. Therefore, substantial amounts of abciximab remain on circulating platelets 8 to 15 days after treatment. The observed degree of receptor occupancy corresponds to GPIIb/IIIa receptor blockade levels of 29% and 13%, respectively. To date, there are no data to suggest the occurrence of platelet redistribution of low molecular weight GPIIb/IIIa receptor antagonists. Whether or not redistribution of the platelet receptor antagonist provides an explanation for the phenomenon of 'passivation' is doubtful.

There are several potential limitations to this study which are worthy of consideration. The decision to use ADP as the sole agonist for determination of ex vivo platelet aggregation was based upon the fact that previously reported clinical studies which assessed the effects of alterations in ionized calcium concentrations upon platelet reactivity were done with ADP as the agonist (Phillips et al., 1997) as is the case with many of the clinical trials of GPIIb/IIIa receptor antagonists (Adgey, 1998). Limiting the aggregation studies to one agonist reduced the amount of blood needed for each sampling period, thereby preventing excessive blood loss over the first 24 h during which time multiple blood samples were taken. One might question the lack of data pertaining to bleeding time determinations. Bleeding time profiling of SM-20302 has been performed previously (Rebello et al., 1998a), and was not included in the present protocol due to ethical concerns in the conscious animals. Perhaps a more compelling reason for not including bleeding time determinations in the present protocol is that bleeding time measurements are viewed as not being highly reproducible (Channing-Rodgers and Levin, 1990). Furthermore, the additional data would not have added significantly to the main thesis of the study since it is well documented that inhibition of the platelet GPIIb/IIIa receptor results in an increase in the bleeding time.

5. Conclusion

In conclusion, SM-20302, in an effective in vivo antithrombotic dose, inhibits ex vivo platelet aggregation completely in citrated platelet-rich plasma, but only partially in heparinized platelet-rich plasma. The presence of a physiologic ionized calcium concentration may enhance the sensitivity of the turbidimetric aggregation method thereby optimizing dosing regimens and monitoring the effects of platelet GPIIb/IIIa receptor blockade. The temporary and partial inhibition of platelets induced by a sole treatment (absence of heparin or aspirin) with SM-20302 compared to controls was associated with a long-term prevention of primary thrombus formation, a reduction in the extent of myocardial infarction and an improved survival over the course of the six-day experimental protocol. The experimental results suggest that SM-20302 may serve as an effective agent for the prevention of arterial thrombotic events.

Acknowledgements

This study was supported in part by the Cardiovascular Pharmacology Research Fund of the University of Michigan. During the tenure of this study, S.S.R. was the recipient of an Advanced Postdoctoral Fellowship from the American Heart Association, Michigan Affiliate.

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