Prevention of Arterial Thrombosis by Intravenously Administered Platelet P2T Receptor Antagonist AR-C69931MX in a Canine Model

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ABSTRACT

P2Y1, P2X1, and P2T receptors mediate ADP-induced platelet aggregation. The antithrombotic effects of AR-C69931MX (N6-[2-methylthio)ethyl]-2-[3,3,3-trifluoropropylthio]-5'-adenylic acid, monoanhydride with dichloromethylenebiphosphonic acid), a selective P2T platelet receptor antagonist, was assessed in a canine model of arterial thrombosis. Placebo or AR-C69931MX (4.0 μg/kg/min for 6 h) pretreatment was administered as an intravenous infusion beginning 15 min before inducing vessel wall injury. A 300-μA anodal current was applied to the intima of the carotid artery for 180 min or discontinued 30 min after cessation of blood flow due to thrombus formation. Each of five control animals developed occlusive arterial thrombosis within 3 h after induction of vessel wall injury. In contrast, carotid artery blood flow in five of six AR-C69931MX-treated animals was maintained for the duration of the protocol. Ex vivo platelet aggregation in response to adenosine diphosphate was inhibited at the first measurement time point of 75 min after the start of drug infusion and remained inhibited during drug administration. Bleeding time values were increased in the drug-treated group. Values for both the ex vivo platelet aggregation and the bleeding times returned to control values shortly after discontinuation of AR-C69931MX. The results indicate that AR-C69931MX antagonizes the ex vivo and in vivo aggregatory actions of ADP, and displays a rapid onset and offset of action with the ability to prevent occlusive arterial thrombus formation. AR-C69931MX may be suitable for the management of patients who require short-term modulation of platelet function.

Understanding the central role of platelet activation, adhesion, and aggregation in formation of arterial thrombi, plus new insights concerning associated molecular mechanisms, provides an incentive for development of site-specific antiplatelet/antithrombotic agents (Adams et al., 1987). An acceptable compound would be one that prevents intravascular platelet-dependent arterial thrombosis without substantially affecting hemostasis.

Adenosine diphosphate (ADP) is present in high concentrations in the dense granules of the platelet and is released during platelet aggregation (Gaarder et al., 1961; Born, 1962). When exposed to ADP, platelets undergo a shape change, and an increase in intracellular calcium secondary to opening of a receptor-operated cation channel. Furthermore, ADP released into the fluid phase serves the function of recruiting platelets during aggregation. The increase in intracellular calcium ions mediates a conformational change and activation of surface glycoprotein IIb/IIIa fibrinogen receptors that form the final common pathway in platelet aggregation.

Theoretically, therefore, a selective antagonist of ADP-induced platelet activation should function as an effective antiplatelet/antithrombotic agent. The availability of selective inhibitors of the platelet ADP receptor provides an opportunity to elucidate the contribution of endogenous ADP to platelet aggregation and thrombus formation in vivo.

The observation that adenosine triphosphate (ATP) functions as a competitive antagonist to ADP (MacFarlane and Mills, 1975) provided the catalyst to define the platelet ADP receptor as the P2T subtype purinoreceptor (Gordon, 1986) localized to the blood platelets. Human platelets possess three ADP receptor subtypes, one coupled to phospholipase C, P2Y1, another to the inhibition of adenylate cyclase, P2T, and a third ionotropic P2X1 receptor leading to the influx of calcium (Daniel et al., 1998). Studies with selective antagonists.
nists indicate that the P2Y1 receptor participates in ADP-induced shape change and aggregation. Inhibition of either P2Y1 or P2T with selective antagonists prevents ADP-induced thromboxane A2 generation, indicating that coactivation of the P2T and P2Y1 receptors is essential for this event (Jin and Kunapuli, 1999). Therefore, the P2T receptor is essential for the full aggregation response to ADP, a conclusion supported by the observation that ADP is unable to induce shape change or platelet aggregation in platelet-rich plasma (PRP) prepared from P2Y1-deficient mice. However, ADP is still able to inhibit adenylyl cyclase in prostaglandin E1-stimulated platelets from the P2Y1-deficient mice (Leon et al., 1999). Occupation of the P2T receptor by ADP leads to inhibition of adenylyl cyclase (Paul et al., 1999). Antagonists that act at the P2T receptor limit the extent of platelet aggregation induced by ADP (Humphries et al., 1994). Thus, a fall in basal cAMP on activation of platelets by ADP may be linked to a reduced rate of Ca2+ reuptake, thus resulting in a sustained aggregation response. A P2T receptor antagonist would limit the decrease of cAMP and Ca2+ reuptake would occur unimpeded, thus reducing or preventing the potential for platelet aggregation.

Although ATP is a competitive antagonist of ADP at the P2T receptor (Gordon, 1986), it is not acceptable as a therapeutic agent due to unfavorable pharmacokinetics in addition to lack of specificity of action. AR-C69931MX showed no significant activity in modifying the response to arterial wall injury by preven-

Materials and Methods

Animal Investigation. The studies conform to the Position of the American Heart Association on Research Animal Use adopted 11 November 1984. The procedures are in accordance with the guidelines of the University of Michigan (Ann Arbor) University Committee on the Use and Care of Animals. Veterinary care is provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association for 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.

Drugs and Reagents. AR-C69931MX was provided by AstraZeneca Research and Development Charnwood, Loughborough, England, as a tetrasodium salt freely soluble in water (batch no. 4256J). The powder was dissolved in 0.9% sodium chloride solution for injection before administration. The dry powder was stored in the dark at a temperature of −10°C to −20°C. All solutions were prepared daily immediately before use. The placebo for AR-C69931MX was 0.9% sodium chloride solution for injection. The placebo and active drug were administered by a continuous intravenous infusion with the aid of a syringe infusion pump. The infusion apparatus was covered with aluminum foil to protect the infusion solutions from light. The drug was delivered at a rate of 4.0 μg/kg/min. The concentration of the solution was adjusted so that the proper amount of drug to be administered over 1 min was contained in 0.1 ml of fluid. The placebo was administered at the same infusion rate of 0.1 ml/min. All other reagents used in this study were obtained from Sigma Chemical Co. (St. Louis, MO).

Model of Arterial Occlusion. The animal model used in this investigation is a modification of one developed by our laboratory for the study of experimentally induced arterial thrombosis. The experimental procedure results in the formation of a platelet-rich intravascular thrombus at the site of an electrolytically induced endothelial lesion in proximity to a distal arterial stenosis. Occlusive carotid artery thrombosis develops in response to vascular injury resulting from application of an anodal current to the intimal surface of the vessel.

Surgical Preparation (Fig. 2). The surgical procedures described in this study were conducted using “clean” surgical techniques. In the present study, all experiments were acute, nonsurvival studies in which the animals were maintained continuously.
under surgical anesthesia and euthanized at the conclusion of the experimental protocol. Euthanasia was induced by anesthetic overdose using sodium pentobarbital and a saturated solution of potassium chloride for induction of cardiac arrest.

Purpose bred, beagle dogs (mean body weight 8.7 kg) (Covance Research Products, Inc., Kalamazoo, MI), of either sex, were anesthetized with sodium pentobarbital (30 mg/kg i.v.), intubated, and ventilated with room air and positive pressure at a stroke volume of 30 ml/kg and a frequency of 12 breaths/min (Harvard Apparatus, South Natick, MA). Cannulae were placed in the right femoral artery for monitoring arterial blood pressure (P23 pressure transducer; Gould Inc., Oxnard, CA) and in the right femoral vein for obtaining blood samples. The left femoral venous cannula was flushed thoroughly with 0.9% sodium chloride solution for injection after drug administration or after obtaining blood samples. The right carotid artery was instrumented with a Doppler flow probe (Transonic Systems, Inc., Ithaca, NY), an indwelling electrode, and a stenosis formed by the placement of a C-shaped nylon ring on the vessel and adjusting a nylon screw that compresses the vessel. The vessel was compressed to the point where the pulsatile pulse in the Doppler flow trace was reduced by 50% without altering mean blood flow. The intraluminal electrode consisted of a 30-gauge, Teflon-insulated, silver-coated, copper wire attached to the tip of a 25-gauge hypodermic needle. The wire and needle tip assembly were inserted through the wall of the artery and positioned in a manner that allowed the uninsulated segment of the electrode to remain in direct contact with the intimal surface of the vessel. Proper positioning of the electrode in the artery was confirmed by visual inspection at the end of each experiment. In addition, visual evidence of an area of vessel wall injury surrounding the electrode needed to be present for the study to be valid. Figure 2 illustrates the manner in which the carotid artery was prepared and instrumented. The external portion of the stimulating electrode and the flow probe wires were secured to the surrounding tissue with sutures.

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 Co., Quincy, MA). The cathode was connected to a distant s.c. site. Application of an anodal d.c. current to the intimal surface of the carotid artery results in a deep, thrombogenic, vascular wall lesion with exposure of subendothelial components. The current delivered to the artery was monitored continuously with an ammeter and was maintained at 300 μA for a period of 3 h or was discontinued 30 min after a stable occlusive thrombus had formed.

Recordings of blood pressure, the lead II electrocardiogram, and mean and phasic blood flow from the carotid artery were obtained on a model 7 polygraph recorder (Grass Instrument Co.). The original tracings from each experiment are recorded on a Grass polygraph and stored on paper. In addition, the electronic data recordings were stored on computer disk using a P-o-ne-ma data acquisition system. At the conclusion of each experiment, the data were entered into spreadsheets on a Macintosh computer and stored on Syquest hard disk with duplicate copies maintained by the principal investigator.

Protocol. The protocol for studies designed to determine the efficacy of AR-C69931MX for the prevention of primary occlusive arterial thrombosis is shown in Fig. 3. It was designed to determine whether AR-C69931MX administered intravenously, in a dose of 4.0 μg/kg/min, will prevent primary occlusive arterial thrombosis. Primary thrombosis is defined as occlusive thrombus formation in response to deep arterial wall injury induced by the application of an anodal current (300 μA for 180 min) to the intimal surface of the carotid artery. The dose of AR-C69931MX (4 μg/kg/min) was based upon previous studies in the Folts’ model of repetitive cyclic flows and on ex vivo inhibition of platelet aggregation. The infusion of AR-C69931MX was started 15 min before application of the anodal current and continued for a total duration of 6 h and 15 min.

The experimental protocol was initiated after completion of the surgical instrumentation of the carotid arteries. The instrumented animals were maintained under anesthesia, as described above. The animals were randomized to receive either 0.9% sodium chloride solution for injection (placebo) or AR-C69931MX intravenously as a continuous infusion.

Fifteen minutes after initiating the infusion of AR-C69931MX or placebo, the anodal current was applied (300 μA) to the intimal surface of the right carotid artery for a maximum period of 3 h or was terminated 30 min after blood flow in the artery had remained stable at zero flow, thereby confirming formation of a stable occlusive thrombus. The objective of this protocol was to determine whether AR-C69931MX could prevent primary occlusive thrombosis or would prolong the time to occlusive thrombus formation. Two groups of animals were studied. Group I served as placebo-treated controls and received the vehicle for the test drug (0.9% sodium chloride solution for injection). Group II received the test drug AR-C69931MX. The

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**Protocol to Assess the Efficacy of AR-C69931MX for Prevention of Primary Arterial Thrombosis**

<table>
<thead>
<tr>
<th>AR-C69931MX</th>
<th>μg/kg/min Infusion</th>
<th>4 hrs</th>
<th>180 min Anodal Current @ 300 μA</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15</td>
<td>0</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>0</td>
<td>60</td>
<td>*</td>
<td>*</td>
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<tr>
<td>120</td>
<td>180</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>240</td>
<td>300</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>360</td>
<td>420</td>
<td>*</td>
<td>*</td>
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</table>

**Instrument carotid artery:** Flow probe, stenosis, anodal electrode, Intravenous catheter for drug administration, Carotid catheter for blood pressure recording, ECG leads

**Monitor:** Blood Pressure, Heart Rate/ECG, Carotid Artery Blood Flow, Bleeding Time, Platelet Aggregation

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Fig. 3. Protocol used for the assessment of AR-C69931MX. The protocol is designed for the purpose of determining drug efficacy for the prevention of primary thrombosis in response to deep vessel wall injury. A 0.9% sodium chloride solution for injection served as the placebo treatment. AR-C69931MX (4 μg/kg/min) was administered during application of the anodal injury current applied to the right carotid artery. The in vivo efficacy of the drug is correlated with ex vivo alterations in platelet reactivity as determined by platelet aggregation studies in citrate anti-coagulated PRP and in which ADP serves as the aggregating agent. Cardiovascular and hematologic parameters were recorded as indicated in the figure (+). Drug or placebo infusions were discontinued after 375 min and the animals were observed for an additional 60 min.
placebo and drug treatment regimens were administered by continuous intravenous infusion.

**Tongue and/or Buccal Bleeding Times.** Bleeding times were determined with the use of a SurgiCut device, which makes a uniform incision 5 mm long and 1 mm deep on the upper surface of the tongue. The tongue lesion was blotted with a filter paper every 30 s until the transfer of blood to the filter paper was no longer apparent. The interval, from the time of the tongue incision until the time that blood is no longer transferred to the filter paper, was recorded as the “tongue bleeding time”. The buccal bleeding time was conducted in a manner similar to that described above with the exception that the SurgiCut-induced lesion was made on the inner surface in the upper buccal region.

**Post-Mortem Examination of the Blood Vessels and Determination of Thrombus Weight.** Upon conclusion of the protocol, the carotid artery was dissected free as far as possible and opened longitudinally. The intravascular position of the anodal electrode was verified and the injury site identified on the intimal surface of the vessel. The thrombus was removed and weighed on an analytical balance.

**Hematologic Measurements/Bleeding Time/Platelet Aggregation.** Complete blood counts (red blood cells, white blood cells, platelets) were done using an H-10 counter (Texas International Laboratories, Houston, TX). Other determinations included blood pressure, heart rate, arterial blood flow, bleeding times, and ex vivo platelet aggregation, which were monitored repeatedly during the experimental protocol as indicated in Fig. 3.

**Ex Vivo Platelet Aggregation Studies.** Blood was taken for whole-blood cell counts and platelet aggregation studies at baseline and at specific time points as indicated in the protocol depicted in Fig. 3. Venous blood (10 ml) was drawn into a plastic syringe containing 3.7% sodium citrate as the anticoagulant [1:10 citrate/blood (v/v)]. The platelet count was determined with an H-10 Counter (Texas International Laboratories). Platelet-rich plasma, the supernatant present after centrifugation of the anticoagulated whole blood at 1000 rpm (140 M) 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 M) for 10 min and discarding the bottom cellular layer.

Ex vivo platelet aggregation was measured by established spectrophotometric methods with a four-channel aggregometer (BioData-PAP-4; BioData Corp., Hatboro, PA) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C. Determinations of ex vivo platelet aggregation were performed with ADP (20 μM). A subaggregatory concentration of epinephrine (550 nM) was added to the PRP to prime the platelets before exposure to the aggregating agent. Values are expressed as percentage of aggregation, representing the percentage of light transmission standardized to PRP and platelet-poor plasma samples yielding 0 and 100% light transmission, respectively.

**Inclusion Criteria.** Animals included in the final protocol met the following pre-established criteria: 1) ability for platelets to aggregate in response to arachidonic acid; and 2) a circulating platelet count of not less than 100,000/μl; 3) demonstrated ability for platelets to aggregate in response to ADP before administration of placebo or AR-C69931MX; and 4) absence of heartworms upon final post-mortem examination. In addition, at the conclusion of the protocol, post-mortem examination was conducted on each carotid artery subjected to electrolytic injury to document proper placement of the intraluminal electrode. Confirmation of proper electrode placement was obtained by gross visual evidence of a lesion on the intimal surface of the affected vessel.

**Double Blind Randomized Study.** The protocols were conducted as a double blind study in which the animals were randomized to either the control or drug-treated groups. Randomization was accomplished by placing coded cards in an envelope. After the animal had been instrumented, a person not associated with the study selected a card from the envelope indicating the treatment regimen to be followed. The person conducting the study was unaware of the treatment being applied.

**Statistical Analysis.** The data are represented as mean ± S.E. A two-way ANOVA (repeated measures) was used to assess differences in platelet aggregation, carotid artery blood flow, hemodynamics, and whole-blood cell counts over time among groups. Fisher’s protected least significant difference and Bonferroni/Dunn post hoc analysis were used to determine significance at P < .05. An unpaired t-test was used 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 < .05. The incidence of occlusion between groups was compared using Fisher’s exact test.

**Results**

AR-C69931MX was examined for its ability to modify or prevent the formation of an occlusive arterial thrombus in response to a deep arterial lesion in the canine carotid artery. Two groups of animals, a placebo-treated (n = 5) and a drug-treated group administered AR-C69931MX (n = 6) were subjected to electrolytic injury of the partially constricted (critical stenosis) right carotid artery. The resulting thrombi were adherent to the injured arterial wall and consisted of a platelet-rich (“white head” and a “red tail”), tapered mass extending from the point of injury distally in the carotid artery. The arterial thrombi were gray-white and friable, and composed of a tangled mesh of platelets, fibrin, erythrocytes, and leukocytes (Romson et al., 1980).

**Carotid Artery Blood Flow.** The data in Table 1 summarize the results relating to the effects of AR-C69931MX on the prevention of carotid artery thrombosis. Thrombotic occlusion of the carotid artery occurred in each of the five control animals. The mean time to occlusion was 105.6 ± 14.9 min. The occlusive thrombus remained stable for the entire duration of the experimental protocol without undergoing spontaneous lysis (Fig. 4). In contrast only one of the six animals in the drug-treated group developed an occlusive thrombus, which occurred at 195 min after application of the anodal current to the intimal surface of the carotid artery. For the purpose of statistical comparison of the times to occlusion, a value of 420 min was assigned to those animals that did not develop an occlusive thrombus at the end of the protocol. Therefore, the mean time to thrombotic occlusion in the animals treated with AR-C69931MX was 382.5 ± 37.5 min.

**TABLE 1**

<table>
<thead>
<tr>
<th>Effect of AR-C69931MX upon primary arterial thrombosis</th>
<th>0.9% Saline (n = 5)</th>
<th>AR-C69931MX (40 μg/kg/min) (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal body weight (kg)</td>
<td>8.8 ± 0.5</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>Time to occlusion (min)a</td>
<td>105.6 ± 14.9</td>
<td>382.5 ± 37.5b</td>
</tr>
<tr>
<td>Incidence of occlusion (%)</td>
<td>100 (5/5)</td>
<td>16.7 (1/6)b</td>
</tr>
<tr>
<td>Thrombus weight (mg)c</td>
<td>47.0 ± 4.7</td>
<td>8.0 ± 3.4b</td>
</tr>
</tbody>
</table>

a For the purpose of statistical analysis, the time to occlusion in five of the six AR-C69931MX-treated animals was considered as 420 min even though vessel patency was present upon termination of the study protocol. One of the animals developed carotid artery occlusion at 195 min after application of the anodal current to the intimal surface of the vessel. b P < .05. c Thrombus size, as determined by weight, was determined at the conclusion of the experimental protocol. A significantly larger thrombus was retrieved from the carotid arteries of control animals, which received a continuous infusion of 0.9% saline.
**Thrombus Weight.** The mean thrombus weight in the carotid arteries of the control animals was significantly greater than the weight of the thrombi removed from the electrolytically injured vessel of the drug-treated animals, 47.0 ± 4.7 versus 8.0 ± 3.4 mg, respectively.

**Post-Mortem Examination of the Carotid Arteries.** A distinct area of deep arterial wall injury was present in the carotid arteries of both the control and AR-C69931MX-treated animals as a result of having been subjected to local application of an anodal current. Despite the presence of extensive vessel wall injury, the intravenous infusion of AR-C69931MX maintained vessel patency during the drug administration with occlusive thrombosis developing in only one of the six animals.

**Bleeding Time Determinations.** Bleeding time determinations were performed by making a standardized lesion on the surface of the tongue as well as on the upper inner lip. The bleeding time in the control animals did not change from baseline when determined repeatedly over the duration of the protocol. In the animals receiving AR-C69931MX, both the tongue and buccal bleeding times increased significantly above baseline when determined at 1 h after commencing the intravenous infusion of AR-C69931MX. The bleeding time data are summarized in Fig. 5 and Table 2. Tongue bleeding times gave a value (minutes) approximately two times that obtained for the buccal bleeding time. There was a further increase in the tongue and buccal bleeding times at 6.25 h after commencing the infusion of AR-C69931MX.

Upon cessation of the drug infusion, both the tongue and buccal bleeding times returned to baseline values during the next 60 min. The latter finding is of interest because bleeding flow in the carotid artery was maintained in five of the six drug-treated animals despite the fact that bleeding times were no longer increased. Thus, bleeding time determinations may not provide an accurate assessment of a drug’s ability to prevent primary occlusive thrombus formation.

**Ex Vivo Platelet Aggregation in Response to ADP.** PRP was prepared from jugular vein venous blood samples obtained at baseline, 75 min, and 375 min after commencing the administration of 0.9% sodium chloride solution for injection or AR-C69931MX. The final blood samples were obtained 60 min after discontinuing the intravenous infusion of placebo or AR-C69931MX. Figure 6 presents a graphic representation of the platelet aggregation responses when ADP was used as the agonist. ADP resulted in near maximal aggregation of platelets in PRP prepared from blood of placebo-treated animals. The intravenous infusion of AR-C69931MX achieved marked inhibition of platelet aggregation in response to ADP within the first 75 min after beginning the drug infusion. The inhibition of ADP-induced platelet aggregation was maintained during the course of the drug infusion. Within 60 min of discontinuing the infusion of AR-C69931MX the platelet aggregation response to ADP had returned to near baseline values.

**Hematologic and Hemodynamic Determinations.** Tables 3 and 4 summarize the hematologic and hemodynamic values in the placebo-treated and AR-C69931MX-treated an-
receptor represents the final common pathway for the forma-
tion of a platelet-dependent thrombus. Pharmacologic interven-
tions that impair receptor function or expression in the ligand-
receptive state have the potential to prevent formation of occlusive arterial thrombi (Coller et al., 1989; Mickelson et al.,
1989). Although effective as inhibitors of platelet function, the
GPIIb/IIIa receptor antagonists coat the platelets and induce a
thrombasthenia-like disorder, and may also cause thrombocy-
topenia. In addition they may cause excessive bleeding, espe-
cially that involving the central nervous system.

Adenine nucleotides affect a number of cellular events in a
wide array of tissues by acting on specific cell receptors that
are classified as P2 purinoreceptors. P2X and P2Y are located
on smooth muscle and other cell types, whereas P2Z is lo-
cated on mast cells and the P2T receptor has a limited cel-
dular distribution; confined to the platelet (Gordon, 1986;
Kennedy, 1990). ADP-dependent platelet aggregation is me-
diated by the P2T purinoreceptor and is inhibited by ATP, a
competitive P2T receptor antagonist. ADP brings about the
exposure of fibrinogen receptors (GPIIb/IIIa), resulting in
platelet aggregation by opening of a platelet receptor-operat-
ed cation channel responsible for the rapid influx of extra-
cellular calcium ion. Although ADP is known to inhibit ade-
nylate cyclase in platelets, the association between this effect
and other actions of ADP remains equivocal because other
agents known to inhibit platelet adenylate neither induce nor
enhance aggregation unless adenylate cyclase is stimulated ini-
tially (Haslam et al., 1978; Cusack and Hourani, 1982c).

The present study describes the in vivo antithrombotic and
ex vivo antiplatelet actions of an ATP analog AR-C69931MX.
ATP undergoes rapid metabolic inactivation, lacks selectiv-
ity, and functions as an agonist at P2 purinoreceptor sub-
types in other tissues. In contrast, AR-C69931MX shows a
high degree of selectivity for the platelet P2T receptor and is
resistant to metabolic degradation by ectonucleotidases present
on the surface of blood cells. The reduced suscepti-
bility of AR-C69931MX to metabolism by ectonucleotidases is
attributed to the presence of the β,γ-methylene link in the
triphosphate chain (Pearson, 1985; Welford et al., 1986). The
mechanism to account for the observed rapid decline in the in
vivo pharmacologic activity of AR-C69931MX upon cessation
of drug infusion remains undefined.

There is general agreement that ADP plays an important
role in the spreading of platelets and contributes to the initial
events in hemostasis (Born, 1962). ADP may be released from
nonactivated platelets, enabling it to act synergistically with
signals generated by adhesion molecules. Thus, ADP partic-
ipates in the early phases of platelet activation, providing a
mechanism by which ADP antagonists may contribute to
inhibition of in vitro platelet aggregation and in vivo modu-

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>1 h</th>
<th>6 h</th>
<th>7 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tongue</strong></td>
<td>0.9% saline</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>AR-C69931MX</td>
<td>1.9 ± 0.2</td>
<td>3.8 ± 0.6</td>
<td>6.4 ± 1.9</td>
</tr>
<tr>
<td><strong>Buccal</strong></td>
<td>0.9% saline</td>
<td>2.1 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>AR-C69931MX</td>
<td>2.2 ± 0.1</td>
<td>7.8 ± 1.2</td>
<td>9.7 ± 2.6</td>
</tr>
</tbody>
</table>

* P < .05 compared with 0.9% saline control group.
* P < .05 compared with AR-C69931MX buccal bleeding time group.
lotion of arterial thrombus formation. Agents that prevent the release of ADP or its ability to activate its platelet receptor may offer advantages over other currently used antiplatelet agents. Three types of receptors, P2Y1, P2X1, and P2T, mediate the effects of ADP on platelets. Although P2Y1 and P2T receptors mediate different signal transduction pathways, both are necessary for full responses involved in platelet aggregation. Whereas the P2Y1 receptor is present on platelets and in blood vessels, the P2T receptor is found only on platelets (Glusa et al., 1999). Thus, antagonists that act at the P2T receptor can limit the extent of platelet aggregation without affecting ADP-mediated vascular responses. The development of AR-C69931MX, a P2T purinoreceptor antagonist possessing high potency and platelet selectivity, provides a new pharmacologic approach for modulating platelet reactivity with the potential to prevent arterial thrombosis.

A consideration of the combined observations consisting of ex vivo platelet aggregation, bleeding time determinations, and carotid artery blood flow provides compelling evidence that AR-C69931MX, a P2T receptor antagonist, has a favorable effect in terms of preventing occlusive thrombus formation in a severely injured artery in the presence of a critical stenosis. When administered as a continuous intravenous infusion (without a loading dose) the onset of the drug response is apparent within the first 75 min as determined by inhibition ADP-induced ex vivo platelet aggregation and modest increases in both the tongue and buccal bleeding times. The duration of drug action is relatively short when assessed on the basis of bleeding time determinations and ex vivo platelet aggregation studies. Upon cessation of drug administration, there is a return to baseline values for both ex vivo platelet aggregation responses to ADP and the tongue and buccal bleeding time values. However, blood flow in the injured carotid artery in five of six animals was sustained in the drug-treated group at the completion of the observation period. The protocol design did not permit a determination of how long blood flow would be maintained after terminating the drug infusion. The continued presence of blood flow in the injured artery, despite the return to baseline for platelet reactivity, may be a result of the time required for formation of an occlusive thrombus once drug administration was terminated. An important question to be answered is whether a prolonged maintenance infusion would preserve arterial blood flow and permit time for vessel "passivation" to occur to the point where the site of arterial wall injury ceased to be thrombogenic. Additional studies in a chronic animal model, as was done with the GP IIb/IIIa receptor antagonist 7E3 F(ab’)2 (Bates et al., 1992) will be necessary to address this question. Continued suppression of platelet reactivity may provide sufficient time for a process by which the injured endothelium “heals over”, thus allowing discontinuation of the platelet inhibitor without progression of thrombus formation despite the presence of a severe vessel wall injury (Groves et al., 1986). It is apparent that ex vivo suppression of platelet responses to ADP can be maintained during infusion of AR-C69931MX and that a rapid recovery of platelet reactivity will occur once administration of the P2T receptor antagonist is discontinued. The “rapid-on” and “rapid-off” kinetics of AR-C69931MX may provide a convenient means of maintaining control of platelet function under clinical conditions that do not require protracted suppression of platelet function. The potential importance of P2T receptor antagonists has been confirmed by a recent publication in which CS-747, an orally effective thienopyridine agent, like clopidogrel and ticlopidine, possesses ADP receptor antagonist properties and antithrombotic activity (Sugidachi et al., 2000). In the latter study, the authors used a rat model of arterio-venous shunt thrombosis in which a heparin-filled polyethylene cannula, containing a silk thread in its lumen, was interposed between the jugular vein and the contralateral carotid artery. Antithrombotic efficacy was assessed on the basis of weight of the thrombus forming on the silk
thread within 30 min. Although the results are in agreement with the present study, it should be noted that the published study involves a model in which “thrombus” formation is not the result of platelet vessel-wall interaction, thus departing from the in vivo environment in which occlusive intravascular thrombosis develop as a result of blood cellular components interacting with an injured region of the arterial wall.

The protocol design permitted a comparison between the tongue bleeding time and the buccal bleeding time methods. Relatively longer bleeding time values are obtained from the tongue compared with that from the buccal mucosa. The difference in bleeding time values may be related to the distribution of the blood vessels and their proximity to the surface at different tissue locations. Despite the difference in the time to bleeding cessation, both the tongue and buccal sites displayed similar directional changes with a return to baseline values when drug administration was discontinued. The significance of the bleeding time changes in the dog with respect to the human clinical application of AR-C69931MX is difficult to ascertain. However, studies in human volunteers given AR-C69931MX in a dose sufficient to inhibit ex vivo platelet aggregation in response to ADP resulted in a modest increase in skin bleeding time (Nassium et al., 1999).

In summary, AR-C69931MX administered as an intravenous infusion (4.0 µg/kg/min) prevents occlusive thrombus formation in response to deep arterial wall injury. Maintenance of vessel patency and an acceptable quality of arterial blood flow coincided with demonstrated ex vivo inhibition of ADP-induced platelet aggregation. The duration of action is brief with a return of ex vivo platelet reactivity to predrug values within 1 h after discontinuing drug administration. Other than a prolongation in bleeding time the continuous infusion of AR-C69931MX was devoid of alterations in hematologic and hemodynamic parameters. The platelet selectivity of a P2T receptor antagonist, AR-69931MX (Leff et al., 1997; Ingall et al., 1999), suggests that the drug may have potential as a therapeutic agent in clinical circumstances in which platelet function must be suppressed for a limited duration (Nassium et al., 1999). The latter report concludes that the separation between ex vivo platelet inhibition and bleeding time may translate into a reduced risk of bleeding without compromising antithrombotic activity. The present study provides an in vivo demonstration for prevention of arterial thrombosis by a P2T receptor antagonist in an animal model involving occlusive thrombus formation due to interaction of blood cellular components with an injured vascular surface. The present results supplement that of a previous study with a related P2T receptor antagonist, ARL-67085, that was noted to inhibit dynamic arterial thrombosis, visualized as cyclic reductions in blood flow in the damaged, stenosed femoral artery of the anesthetized dog.

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