Glycoprotein IIb/IIIa Receptor Antagonist CRL42796, in Combination with Aspirin and/or Enoxaparin, Prevents Coronary Artery Rethrombosis after Successful Thrombolytic Treatment by Recombinant Tissue Plasminogen Activator (rt-PA) ¹

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Running Title: In Vivo Antithrombotic Activity of CRL42796

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Nonstandard abbreviations: aPTT, activated partial thromboplastin time

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Abstract

The antithrombotic effect of the GPIIb/IIIa antagonist, CRL42796, (2S)-2-[(2-naphthyl-sulfonyl)amino]-3-[(2-(4-(4-piperidinyl)-2-[2-(4-piperidinyl) ethyl] butanoyl)amino)acetyl] amino)propanoic acid dihydrochloride, administered alone, or in combination with aspirin, and/or enoxaparin, was examined in a canine left circumflex (LCX) coronary artery rethrombosis model. The electrolytic induction of arterial thrombosis was followed by intracoronary rt-PA administration to achieve thrombolysis, and the adjunctive therapy was initiated 15 min earlier and maintained for four hours. Thirty five purpose bred beagle dogs were randomized to receive one of the following treatments: Group 0 (n=6, placebo); Group 1 (n=6, CRL42796 15 µg/kg i.v. loading dose followed by 0.31 µg/kg/min i.v. infusion), Group 2 (n=6, aspirin 7 mg/kg, administered orally, at -47, -23, -17hr before entry into the experimental protocol); Group 3 (n=6, aspirin + CRL42796); Group 4 (n=6, aspirin + enoxaparin 0.6 µg/kg i.v. loading dose followed by 6.0 µg/kg/min i.v. infusion); Group 5 (n=5, aspirin + CRL42796 + enoxaparin). The incidence of LCX reocclusion was: Group 0 - 6/6, Group 1 - 3/6, Group 2 - 5/6, Group 3 - 2/6, Group 4 - 2/6, and Group 5 - 0/5. Aspirin pretreatment increased the tongue-bleeding time, while the addition of CRL42796 or enoxaparin did not prolong bleeding time to a further degree. However, the combination of the three drugs did increase bleeding time significantly, from 173.9±19.8 to 620.0±98.7 sec. In conclusion low-dose CRL42796 together with aspirin and enoxaparin prevented coronary artery rethrombosis although bleeding time was prolonged. The latter may be of concern in the clinical use of combination therapy.
Intravascular thrombosis is the most frequent cause of acute myocardial infarction and thrombolytic therapy has become the mainstay for patient management. Unfortunately, in the absence of adjunctive therapy, a significant number of the patients develop reocclusion after successful thrombolysis in the infarct related artery (Rebello, 1999). Therefore, the concomitant use of adjunctive agents to prevent rethrombosis after coronary angioplasty or successful thrombolysis is needed to maintain vessel patency in patients undergoing revascularization. Platelet adhesion to exposed subendothelial surfaces of injured vessels with subsequent activation and platelet aggregation are known to be involved in rethrombosis. Current clinical practice makes use of one or more “antiplatelet” agents such as aspirin, ticlopidine, clopidogrel, used alone or in combination. Although having a beneficial effect, the various treatment regimens are only partially effective due to their limited platelet inhibitory effects.

The aggregation of platelets is mediated by activation of the platelet membrane glycoprotein IIb/IIIa receptor (GPIIb/IIIa) (Plow and Ginsburg, 1989; Peerschke, 1985). Platelet aggregation can be induced by activation of different membrane receptors such as PAR1, PAR4, P2X1, and P2Y12. Aspirin is effective ex vivo in preventing arachidonic acid-induced platelet aggregation, but lacks significant antiaggregatory efficacy against other platelet agonists (5-HT, epinephrine, thrombin, plasmin, etc.). However, the final common pathway for platelet activation is the surface expression of GPIIb/IIIa and subsequent cross-linking with soluble fibrinogen. Pharmacological blockade of the GPIIb/IIIa, will interfere with the final common pathway for arterial thrombus formation irrespective of the mode of platelet activation. Agents directed at the platelet GPIIb/IIIa receptor preserve vessel patency and adequate blood flow to maintain tissue viability (Makkar et al., 1997). The first platelet GPIIb/IIIa receptor antagonist was the chimeric
7E3 (ReoPro) (Coller et al., 1985). Several antiplatelet agents (abciximab, eptifibatide, tirofiban, ticlopidine, clopidogrel), having mechanisms of action differing from that of aspirin, show efficacy in reducing the incidence of thrombotic occlusion after restoration of arterial blood flow (Spinler et al., 2001). The major drawback to current small molecule GPIIb/IIIa receptor antagonists (tirofiban, eptifibatide) is the need to be administered by the parenteral route and their relatively short pharmacological half-life. Although orally effective GPIIb/IIIa receptor antagonists have been developed and studied clinically, none have achieved the status of approval due to problems related to bioavailability, an effective oral dosing regimen and potential for excessive bleeding.

Potential advantages of a new small molecule GPIIb/IIIa platelet receptor antagonist, CRL42796, are that it is effective by parenteral as well as oral administration. CRL42796 is a non-peptide, bispiperidine, GPIIb/IIIa platelet receptor antagonist. In vitro studies indicate that CRL42796 exhibits a high potency (10^{-8}M) in preventing platelet reactivity in human, monkey, dog, and guinea pig platelets. Previous studies from our laboratory suggested that CRL42796 is effective in maintaining vessel patency with a minimum risk of bleeding when used as the singular antiplatelet agent. It is anticipated that the risk for bleeding may be reduced further by combining CRL42796 with one or more adjunctive agents that act at different sites in the coagulation cascade. Furthermore, the combined use of adjunctive therapies may serve to enhance the efficacy of thrombolysis while at the same time providing a high quality of arterial blood flow.
The potential for excessive bleeding after the administration of a GPIIb/IIIa platelet receptor antagonist, especially in the presence of a thrombolytic agent, presents an additional problem in patient management. Therefore, our recent efforts have focused on the concomitant use of a suboptimal dose of CRL42796, along with aspirin (current standard of care) in the absence or presence of low doses of a low molecular weight heparin, enoxaparin. It was hypothesized that the combined therapy would be more effective in terms of maintaining vessel patency while limiting the potential for excessive bleeding.

Methods:

Animal Investigation

This study conforms to the position of the American Heart Association on research animal use adopted November 11, 1984, by the American Heart Association. The procedures followed in this study were in accordance with the guidelines of the University of Michigan (Ann Arbor) Committee on the use and care of animals, and with the Guide for Care and Use of Laboratory Animals, US Department of Health, Education, and Welfare Publication No. NIH 78-23. Veterinary care is provided by the University of Michigan for Laboratory Animal Medicine.

Reagents

CRL42796 was provided by Research and Development Center of Cephalon France (Maisons-Alfort, FRANCE). γ-Thrombin was purchased from Enzyme Research Lab, IN. Sodium citrate, ADP, arachidonic acid, epinephrine, and other standard reagents were purchased from Sigma Chemical Co.
Model of Vessel Occlusion

Healthy, male and female purpose-bred beagle dogs (9 to 13 kg) were anesthetized with sodium pentobarbital (30 mg/kg, IV), intubated, and ventilated with room air at a tidal volume of 30 ml/kg at a rate of 12 breaths per minute (Harvard Apparatus Inc., Holliston, MA). Catheters were inserted into both the right and left femoral veins and the right jugular vein for blood sampling and drug administration, respectively. Arterial blood pressure was monitored from the cannulated femoral artery with a Millar™ Mikro-tip catheter (Millar Instruments, Inc., Houston, TX). The standard limb lead II of the electrocardiograph was recorded continuously and used to monitor heart rate.

The model used in this study was a modification of one developed by our laboratory for the study of experimentally induced coronary artery thrombosis (Rote, 1994). The model makes use of anodal current-induced electrolytic injury to the intimal surface of the arterial wall that invariably results in the formation of an intravascular thrombus. The heart was exposed by a left thoracotomy in the fifth intercostal space and suspended in a pericardial cradle. A calibrated flow probe (model 1.5RB907, Transonic Systems Inc, Ithaca, NY) was placed on the left circumflex coronary artery to continuously monitor blood flow (ml/min). Securing a suture-ligature around the artery and an adjacent 18-gauge hypodermic needle and then removing the needle produced an external stenosis. An intracoronary electrode was fashioned from the tip of a 25-gauge (4 mm) hypodermic needle attached to a 30-gauge Teflon-insulated, silver-coated copper wire. The intravascular electrode was connected to the positive pole (anode) of a dual-channel square wave generator (Grass S88 stimulator and a Grass Constant Current Unit, model CCUIA, Grass Instrument Co.). The cathode was connected to a distant subcutaneous site. The
current delivered to the artery was monitored continuously on with an ammeter and maintained at 150mA. Proper positioning of the electrode in the vessel was confirmed by visual inspection at the conclusion of each experiment. The arterial wall lesion and subsequent formation of an occlusive thrombus results from direct application of an anodal direct current to the endothelial surface of the vessel leading to exposure of proaggregatory subendothelial elements (Mickelson et al., 1990). The resulting thrombus consists of a platelet-fibrin mass adherent to the vessel wall at the site of intimal injury (Romson et al., 1980) and possesses the morphologic features of acutely formed thrombi found in patients with acute myocardial infarction (Falk, 1985).

**Experimental Protocol**

The present study was designed to assess the ability of CRL42796 to prevent rethrombosis after successful thrombolysis and to determine if a subtherapeutic dose of CRL42796 (15 µg/kg iv loading dose followed by an intravenous infusion of 0.31 µg/kg/min) could prevent rethrombosis when combined with other adjunctive agents such as aspirin, enoxaparin or both.

The proposed studies are outlined in Figure 1 and were conducted in the anesthetized animal in which the coronary artery was subjected to electrolytic injury leading to the formation of a stable occlusive, platelet rich thrombus. The occlusive thrombus was allowed to age for 60 minutes before initiating thrombolytic therapy. The thrombolytic agent, rt-PA was administered as a local injection immediately proximal to the occlusive thrombus 15 min after the initiation of adjunctive therapy. The locally administered lytic agent was given as a slow intraarterial injection of 0.2 mg/kg over 3-4 minutes followed by a continuous infusion of 0.72 mg/kg delivered over a period of 60 minutes. The experiment was terminated at 4 hours after the
initiation of intracoronary administration of rt-PA. The adjunctive therapy was maintained throughout the duration of the experimental protocol.

Thirty-five dogs were randomized among six different groups as follows: Group 0 (n=6) received a placebo i.v. infusion as the adjunctive therapy; Group 1 (n=6) received CRL42796 i.v. loading-dose of 15 µg/kg followed by an intravenous infusion of 0.31 µg/kg/min, Group 2 (n=6) pretreatment with three doses of oral aspirin (7 mg/kg) at -47, -23 and -17 hr and received placebo i.v. infusion on the day of the experiment; Group 3 (n=6) also received aspirin pretreatment and was given CRL42796 i.v. loading dose of 15 µg/kg followed by an infusion of 0.31 µg/kg/min i.v. infusion; Group 4 (n=6) received aspirin pretreatment and was administered enoxaparin 0.6 µg/kg i.v. loading dose followed by 6.0 µg/kg/min i.v. infusion; Group 5 (n=5) was pretreated with aspirin and received CRL42796 and enoxaparin i.v. infusion simultaneously.

Blood samples were obtained at prescribed intervals to assess the coagulation parameters. The mean arterial blood pressure, heart rate and LCX coronary artery blood flow were monitored throughout the experiment. Time to thrombolysis was determined as well as the duration of vessel patency. The thrombolytic agent, rt-PA, was administered via an indwelling cannula positioned immediately proximal to the occlusive thrombus. The lytic agent was administered as a loading dose followed by an infusion fifteen minutes after the initiation of the adjunctive therapy. The local administration of rt-PA limits the total amount of lytic agent required to achieve clot lysis thereby limiting the development of a systemic lytic effect. At the end of the protocol, all animals were euthanized by electrical induction of ventricular fibrillation and the injured vessel segment proximal and distal to the point of injury was removed without disturbing
the intravascular thrombus. The vessel segments were opened longitudinally to allow removal of the intact thrombus. The weight of each thrombus was determined using an analytic balance.

**Quantitative Analysis of Oscillatory Flow**

Criteria were established to quantify the quality of coronary artery blood flow and applied every 30 minutes after application of the anodal injury current. The established criteria are as follows: (1) A rating of 0 indicates the absence of blood flow. (2) A rating of 1 is applied when flow declines to 0 and is restored spontaneously. (3) A rating of 2 indicates a decline in blood flow that is restored spontaneously before it reaches zero flow. (4) A rating of 3 is applied when the blood flow is maintained relatively constant in a non-oscillatory manner (Sudo et al., 1996).

**Inclusion Criteria**

Animals included in the final protocol satisfied the following pre-established criteria: (a) A circulating platelet count of not less than 100,000 per ml. (b) Demonstrated ability for epinephrine-primed platelets to aggregate in response to ADP or arachidonic acid before administration of saline or the candidate agonist. (c) Absence of heart worms and intestinal nematodes upon final post-mortem examination.

**Hematologic Measurements**

Determinations of the aPTT, tongue-template bleeding time, and *ex vivo* platelet aggregation were made at baseline and repeated at 60 min after thrombotic occlusion, 15, 75, 195 and 255 min after *i.v.* administration of placebo, CRL42796, Enoxaparin, or both of the drugs. Blood (10ml) was withdrawn from the right femoral vein cannula into a plastic syringe containing 3.7%
sodium citrate as the anticoagulant (1:10 citrate to blood [vol/vol]) for the ex vivo platelet aggregation determinations. An additional 10 ml of blood was withdrawn into another syringe containing 50 IU heparin. The whole blood cell count was determined with an H-2000 cell counter (Texas International Laboratories, Inc). Platelet-rich plasma (PRP), the supernatant present after centrifugation of whole blood at 660 rpm for 8 min, was diluted with saline to achieve a platelet count of ~200,000/ml. Platelet-poor plasma (PPP) was prepared by centrifuging the remaining blood at 12000g for 10 min and discarding the bottom cellular layer. Ex vivo platelet aggregation was assessed by established spectrophotometric methods with the use of a 4-channel aggregometer (BioDataPAP-4, Bio Data Corp.) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C. Aggregation was induced with ADP (20µmol/L or 5µmol/L), AA (0.65mmol/L) or γ-thrombin (25nmol/L). A subaggregatory concentration of epinephrine (550 nmol/L) was used to prime the platelets before addition of the agonists to induce platelet aggregation. Values for platelet aggregation are expressed as percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively.

The anticoagulation state of the animals was assessed by determining aPTT with a Hemochron™ Analyzer (Technidyne, Edison, NJ) and reagents supplied by the manufacturer. Citrated whole blood (2 ml) was used for these determinations. The bleeding time was measured with a Simplate™ device (Simplate-R, Organon Teknika Corp., Durham, NC) by making incisions of 5 mm long and 1 mm deep on the upper surface of the tongue. The tongue lesion was blotted with a filter paper every 15 seconds until the transfer of blood to the filter paper ceased. The time
from the initial tongue incision to the cessation of active bleeding was recorded as the bleeding
time.

**Statistical Analysis**

The data are expressed as mean±SEM. The data were analyzed by one-way analysis of variance
for group comparisons and for repeated measures followed by a Dunnett post hoc t-test to
determine the level of significance. A paired t-test is used to assess the differences over time
within a group. Vessel flow patency is compared by a Chi square test. Values are considered to
be statistically different at a level of $P < 0.05$. 
Results

Hemodynamic Variables

Thirty-five dogs were entered in this study and randomized among six groups. Mean arterial blood pressure (MABP), hear rate (HR), and rate pressure product (RPP) were not different across groups at baseline. The MABP and RPP decreased throughout the experiment in the control group. CRL42796, enoxaparin alone or together with aspirin pretreatment had no further effect on these parameters during the experiment. However, the combination of the three drugs, aspirin, CRL42796, and enoxaparin, led to a significant lowering of BP and RPP after four hours intravenous infusion (Table 1). The effects on MABP and RPP were most likely related to blood loss from the surgical wound sites and the need to obtain multiple venous blood samples. Fluid replacement was not used for the purpose of expanding the vascular volume.

Blood Coagulation Parameters

Percentage platelet aggregation responses to AA, ADP or γ-thrombin did not change throughout the experiment in the Controls (Group 0). Platelet aggregation induced by AA was inhibited significantly in all of the aspirin pretreated animals, whereas aspirin pretreatment alone did not affect ex vivo platelet aggregation in response to ADP or γ-thrombin. CRL42796 alone caused significant inhibition of ex vivo platelet aggregation responses to AA, ADP and γ-thrombin only in citrated blood, whereas the responses to the agonists were partially decreased in PRP prepared from blood anticoagulated with heparin. Enoxaparin treatment inhibited platelet aggregation in response to γ-thrombin, but did not affect the aggregation responses to ADP or arachidonic acid. However, in the presence of each of the three adjunctive, platelet aggregation responses to each
of the agonists was inhibited significantly in both the citrate and heparin anticoagulated PRP preparations (Table 2, 3).

The aPTT determinations were not altered by any of the adjunctive interventions used alone or in combination when examined throughout the entire experimental protocol. Aspirin, CRL42796 or enoxaparin, used individually, did not increase the tongue bleeding time. In the groups that received aspirin pretreatment plus CRL42796 or enoxaparin, the bleeding time increased moderately when determined one hour after the intracoronary artery administration of rt-PA. Despite the initial increase in the bleeding time, the values returned almost to baseline two hours after discontinuation the rt-PA infusion. However, in the final group receiving rt-PA plus the three adjunctive agents concomitantly, the tongue bleeding time was prolonged significantly throughout the experimental protocol (Table 4). The increase in bleeding time represents the summation of the individual interventions upon the coagulation cascade and alteration of platelet reactivity and cannot be attributed exclusively to any one of the administered agents.

**Time to Thrombolysis, Total reperfusion Time, and Thrombus Weight**

The time to thrombolysis after local administration of rt-PA was similar in each of the six groups, suggesting that pretreatment with the adjunctive agents used alone or in combination did not influence the lytic action of rt-PA.

Aspirin alone decreased the thrombus weight, although the difference compared to the Control Group did not achieve statistical significance. CRL42796 alone, the combination of aspirin with CRL42796 or aspirin plus enoxaparin was associated with a significant decrease of thrombus
weight. The combined administration of the three adjunctive agents caused a further decrease of thrombus weight (Figure 2).

The total reperfusion time or vessel patency, defined as blood flow >20% of baseline blood flow, is shown graphically in Figure 3. Aspirin or CRL42796 alone, or aspirin plus enoxaparin increased the duration of reperfusion compared to the Control Group, although the difference was not statistically significant. However, aspirin plus CRL42796, in the absence or presence of enoxaparin, caused a significant increase in total reperfusion time, while the combination of the three adjunctive agents significantly prolonged the total reperfusion time.

**LCX Flow Patency and Reocclusion Incidence**

A patency score of 3 was assigned to all of the vessels before application of the electrolytic injury current. In each case the patency score achieved a value of 0 at the time of vessel occlusion due to formation of an occlusive arterial thrombus. Approximately 45 min after cessation of blood flow in the LCX coronary artery, each animal received the respective pretreatment regimen followed 15 minutes later by the local, intracoronary administration of rt-PA. Pretreatment with the selected adjunctive agents neither induced clot lysis nor influenced the efficacy of rt-PA, which brought about clot lysis in each of the animals.

In the Control Group, rt-PA administration resulting in a flow patency score of 1 that reverted to 0 within 60 minutes. In contrast, Group 5 animals, which received ASA + enoxaparin + CRL42796, achieved a coronary artery patency score of 3 within 60 min of having received rt-
PA. The flow patency score of 3 was maintained for the duration of the study although this was associated with an increase in the tongue bleeding time.

All the other groups that received different pretreatment regimens, the LCX flow patency score was intermediate between those of the Control Group and Group 5 (Figure 4). Whereas each of the vessels in the Control Group reoccluded by the end of the experimental protocol, none of the injured vessels reoccluded in Group 5. The incidence of LCX reocclusion for each of the six groups is summarized in Table 5.
Discussion

Occlusive arterial thrombosis involves multiple mediators (e.g., fibrinogen, thrombin, ADP, thromboxane A2, collagen, etc.) that contribute to platelet activation and aggregation (Marcus, 1984). The final common pathway in thrombus development involves platelets binding to fibrinogen via the GPIIb/IIIa receptor. Therefore, platelet GPIIb/IIIa receptor antagonists prevent platelet dependent arterial thrombus formation regardless of the inciting agonist. Currently approved GPIIb/IIIa antagonists include the monoclonal antibody Fab fragment abciximab, cyclic peptides based on RGD or related motifs including eptifibatide, and the RGD-based peptidomimetic, tirofiban (Bennett, 2001). Several oral GPIIb/IIIa antagonists (xemilofiban, sibrafiban, lamifiban and roxifiban) were examined experimentally as well as clinically but have not been approved for clinical use due to limited oral bioavailability and the potential to induce serious bleeding.

The non-peptide GPIIb/IIIa receptor antagonist, CRL42796 was reported to prevent primary thrombosis when administered intravenously (Hennan et al., 2002) or after oral dosing (Hennan et al., 2003). The minimal effective intravenous dosing regimen for preventing arterial thrombosis required a loading dose of 15 µg/kg and a continuous infusion ranging between 0.31 and 0.69 µg/kg/min. Our previous study demonstrated the in vivo efficacy of CRL42796 in an electrolytic injury model of the canine carotid and coronary artery. With the lower dose, a progressive decline in blood flow was observed over the duration of the experimental protocol.

In the present study, we determined the minimal effective dose of CRL42796 for prevention of rethrombosis (secondary thrombosis) after successful thrombolysis. The results indicate that
CRL42796, administered in a subtherapeutic dose, was of limited efficacy in preventing arterial rethrombosis after thrombolysis.

*Ex vivo* platelet responses from citrate anticoagulated whole blood did not correlate with the inhibition of arterial thrombosis as citrate chelates ionized calcium (Phillips et al., 1997; Rebello et al., 1998). Ionized calcium is essential for the formation of the GPIIb/IIIa heterodimer complex (Lam, 1992) and for the interaction between the GPIIb/IIIa receptor and fibrinogen (Steiner et al., 1989). Binding of the GPIIb/IIIa receptor antagonist is enhanced when the Ca\(^{+2}\) concentration of platelet rich plasma is decreased by citrate, thus overestimating the efficacy of an antiplatelet agent. In animals treated with the low dose of CRL42796 (0.31 µg/kg/min), rethrombosis was not prevented and the platelet response in PRP prepared from heparin anticoagulated whole blood was reduced, but not inhibited significantly. Thus, in the low dose CRL42769 treatment group, only two of six vessels remained patent throughout the study protocol. Two-thirds of the animals exhibited LCX coronary artery rethrombosis despite the fact that a significant degree of *ex vivo* platelet aggregation was noted when the assay was done in citrate anticoagulated samples.

Aspirin, an irreversible, noncompetitive inhibitor of platelet cyclooxygenase and thromboxane A\(_2\) formation (Smith et al., 1971) is effective in secondary prevention of thrombosis in patients with atherosclerosis (Henneken, 1990; Koller, 1991) and is the standard therapy against which other agents are judged. In the present study, aspirin 7 mg/kg, was administered orally as a pretreatment -47, -23 and -17 hours before commencing the experimental protocol. This dosing regimen of aspirin inhibits platelet responses to arachidonic acid while allowing endothelial
sources of cycloxygenase-2 to regenerate and participate in the synthesis of prostacyclin (PGI2) (Hennan et al., 2001). Animals treated with aspirin were not protected from rethrombosis as each of the coronary arteries reoccluded after successful thrombolysis. The limited efficacy of aspirin may relate to its inability to prevent platelet responses to ADP, thrombin, and collagen plus the added prothrombotic actions of plasmin generated as a result of rt-PA (Topol, 2000). Enhanced protection against rethrombosis was observed among the animals administered aspirin and CRL42796. Total reperfusion time in the aspirin+CRL42796 treated group was longer when compared with animals in which each drug was administered separately. In animals treated with the lower infusion dose of CRL42796 (0.31 µg/kg/min), ex vivo platelet responses in PRP prepared from heparin anticoagulated whole blood were reduced, but not inhibited significantly. Therefore, the addition of aspirin to the treatment regimen with the subsequent inhibition of thromboxane A2 synthesis, provides an added degree of in vivo platelet inhibition.

Enoxaparin, a low molecular weight heparin, is approved for the management of patients with unstable angina. Arterial thrombosis involves the activation of both the coagulation cascade and circulating platelets and the combination of an anticoagulant directed against the formation of thrombin along with an antiplatelet agent may provide a means to maintain coronary artery patency after thrombolysis. The enoxaparin-antithrombin III complex inhibits fXa thereby reducing the activation of the intrinsic coagulation pathway and subsequent generation of thrombin. In the present study, the combination of enoxaparin and aspirin prolonged total reperfusion time, but did not prevent secondary occlusive thrombus formation after successful thrombolysis. Whereas enoxaparin and unfractionated heparin can modulate thrombin formation, their ability to inhibit platelet activation is limited. However the combination of
aspirin + enoxaparin + low dose CRL42796 was effective in preventing secondary thrombosis in each of the animals studied. All the vessels remained patent and the patency score for LCX coronary artery blood flow was similar to that observed before the induction of vessel wall injury. The findings in this study are consistent with clinical observations in which the combined use of a lytic agent with abciximab or eptifibatide has yielded high frequencies of infarct vessel patency (Topol, 2000).

After fibrinolytic therapy there remains a significant area of vessel wall injury due to electrolytic injury (experimental model) or as in the case of a clinical setting there is the continued presence of a fissured atherosclerotic lesion. In both the experimental and clinical settings a prothrombotic vessel surface continues to exist. Added to this is the additional complicating fact that plasmin dependent lytic agents are prothrombotic (Ervin and Peerschke, 2001). The mechanism of platelet activation or inhibition by plasmin is poorly understood. Plasmin at a concentration in excess of 1 caseinolytic unit / ml triggers platelet aggregation at 37°C through direct or indirect activation of the integrin glycoprotein IIb/IIIa (Rahi-Sabile and Pidard, 1995; Loscalzo et al., 1995). Plasmin may promote platelet activation by cleaving the seven transmembrane thrombin receptor to expose the tethered ligand containing the SFLLRN sequence, which directly activates platelets in the same manner as thrombin (Kuliopulos et al, 1999). Additional observations demonstrate a role for secreted dense granule adenosine diphosphate in the potentiation of high-dose plasmin-induced platelet aggregation via the P2Y12 receptor (Ishii-Watabe et al., 2000).
Bleeding times have been reported to be an unreliable measurement [Channing-Rodgers and Levine, 1990]. In the present study, tongue-bleeding times were well correlated with the inhibition of platelet activity and thrombosis. Pretreatment with aspirin caused a slight increase of baseline bleeding time compared with the control group. At the current low dose of CRL42796 we did not observe a significant increase in bleeding and rethrombosis was only partially prevented. The infusion of enoxaparin also did not increase bleeding time and rethrombosis was only partially prevented. In the last group, treated with aspirin, CRL42796 and enoxaparin, rethrombosis was prevented while a significant increase in tongue bleeding time was also observed. The results suggest that bleeding time may be a useful measure for the ability of a platelet receptor antagonist or an anticoagulant to inhibit arterial thrombosis. Inhibition of one or more factors in the intrinsic coagulation cascade will cause an increase the aPTT. Enoxaparin inhibits factor X and increased aPTT, while aspirin and CRL42796 were without effect on aPTT. Of importance is the observation that plasmin-induced aggregation is not inhibited by aspirin or ADP antagonists (Ervin and Peerschke, 2001).

In conclusion, the use of a *subtherapeutic dose* of the platelet GPIIb/IIIa receptor antagonist, CRL42796, was not effective in preventing reocclusive coronary arterial thrombosis or *ex vivo* platelet reactivity. However, the combination of aspirin, enoxaparin and CRL42796 prevented coronary artery rethrombosis after successful thrombolytic therapy. Deep vessel wall injury leads to the formation of a platelet rich “white thrombus”. The latter serves as a nidus that becomes surrounded by the fibrin rich “red thrombus”. Plasmin-dependent lytic agents are effective in bringing about dissolution of fibrin strands making up the “red thrombus”. However, the ability of plasmin to activate platelets facilitates local platelet aggregation along with
exposure of thrombin previously embodied within the “red thrombus”. Thrombin is an effective activator of platelet reactivity, which in concert with plasmin-induced platelet activation promotes an intense prothrombotic environment. Combined use of several pharmacologic interventions, each targeting a different component of the occlusive thrombus, may represent a means by which successful thrombolysis can be achieved while reducing the incidence of rethrombosis. A limitation of the approach is the potential for serious bleeding. The favorable pharmacodynamic properties of CRL42796 of rapid onset of platelet inhibition and relatively short duration of action may provide a greater margin of safety over currently available GPIIb/IIIa receptor antagonists. The present study results provide evidence for the ability of CRL42796 to maintain a favorable quality of blood flow when used in conjunction with aspirin and enoxaparin. Future clinical trials using combination therapy will be needed to determine the safety and efficacy of a combined dosing regimen.
References


platelet aggregation by reductions in the concentration of ionized calcium. *Circulation* 96:1488-1494.


Footnotes:

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Figure Legends

**Figure 1.** Experimental protocol to assess the synergistic effect of combining aspirin and enoxaparin with CRL42796 in a canine model of arterial thrombosis and thrombolysis. The recorded hematological parameters are listed.

**Figure 2.** The weight of residual thrombi removed from the injured coronary vessels in animals from each of the six respective groups. The data are expressed as mean±SEM. When compared with the control group means p<0.05* or p<0.01**.

**Figure 3.** Total reperfusion time in each of the six experimental groups: The data are expressed as mean±SEM. When compared with the control group means p<0.05* or p<0.01**.

**Figure 4.** Left circumflex coronary artery flow patency change in different groups. The quality of coronary artery blood flow was quantified using a scale of 0-3 as described in the Methods and described by Sudo and Lucchesi, 1996. The individual data points and SEMs (60 min intervals) were omitted from the graphic display of the data for the purpose of clarity. The actual means and SEMs for each group at the various 60 min time points are presented below the graph.
Table 1:

Changes in Heart Rate (HR), Mean Arterial Blood Pressure (MABP), and Rate Pressure Product (RRP)

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<th>Group 0</th>
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<td>160.7±6.4</td>
<td>161.0±11.8</td>
<td>154.0±12.9</td>
<td>167.3±10.0</td>
<td>149.0±12.1</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>178.5±11.1</td>
<td>171.3±9.1</td>
<td>175.3±12.3</td>
<td>178.8±11.3</td>
<td>176.5±9.8</td>
<td>149.0±23.6</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>175.5±4.8</td>
<td>163.0±14.8</td>
<td>163.7±11.0</td>
<td>156.8±13.5</td>
<td>168.0±16.0</td>
<td>171.3±16.4</td>
</tr>
<tr>
<td>MABP</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>117.2±12.4</td>
<td>115.7±10.9</td>
<td>147.2±14.2</td>
<td>131.0±22.5</td>
<td>130.1±11.7</td>
<td>99.0±10.6</td>
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<tr>
<td>Pre-tPA</td>
<td>92.4±19.9</td>
<td>95.5±17.7</td>
<td>106.2±14.6*</td>
<td>102.7±19.7</td>
<td>123.8±11.4</td>
<td>77.7±11.5</td>
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<td>4hr-tPA</td>
<td>68.5±17.0</td>
<td>84.1±15.1</td>
<td>83.2±15.8</td>
<td>88.3±13.2</td>
<td>63.2±12.8**</td>
<td>39.2±13.0**</td>
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<td>RPP</td>
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<tr>
<td>Baseline</td>
<td>192.6±20.5</td>
<td>189.3±23.0</td>
<td>237.5±27.3</td>
<td>207.8±44.6</td>
<td>218.3±24.9</td>
<td>148.7±22.6</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>166.0±41.1</td>
<td>169.4±35.9</td>
<td>191.6±33.4</td>
<td>187.0±39.9</td>
<td>217.3±21.1</td>
<td>119.1±27.9</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>119.3±29.7</td>
<td>146.6±31.8</td>
<td>141.4±31.3</td>
<td>113.8±28.8</td>
<td>103.0±21.3*</td>
<td>69.2±23.2</td>
</tr>
</tbody>
</table>

Group 0 (n=6) received placebo (saline) i.v. infusion; Group 1 (n=6) treated with CRL42796 i.v. 15 µg/kg followed by 0.31 µg/kg/min i.v. infusion; Group 2 (n=6) pretreated with three doses of oral aspirin 7 mg/kg at -47, -23 and -17 hr then a placebo i.v. infusion on the day of experiment; Group 3 (n=6) received oral aspirin pretreatment and was given CRL42796 i.v. 15 µg/kg followed by 0.31 µg/kg/min i.v. infusion; Group 4 (n=6) received oral aspirin pretreatment as above. On the day of the study Group 4 animals received an initial dose of enoxaparin 0.6 µg/kg i.v. followed by an i.v. infusion of 6.0 µg/kg/min; Animals in Group 5 (n=5) were pretreated with aspirin and simultaneously received CRL42796 and enoxaparin i.v. infusion.
Data represent mean±SE. HR, heart rate; MABP, mean arterial blood pressure; RPP, rate pressure product. When the values from different time points compared with baseline values within each group, * indicates p<0.05; ** indicates p<0.01.

**Table 2**

*Ex Vivo* Platelet Aggregation Responses to Arachidonic Acid (AA, 0.65 mM), ADP (5 and 20 µM), and Gamma-Thrombin (γ-TB, 25 nM)*

<table>
<thead>
<tr>
<th></th>
<th>Group 0</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AA (0.65 mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>64.8±8.0</td>
<td>56.8±10.4</td>
<td>7.8±2.2##</td>
<td>9.0±2.4##</td>
<td>18.4±6.4##</td>
<td>27.8±9.2##</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>64.8±6.9</td>
<td>20.0±10.5*</td>
<td>18.0±8.8</td>
<td>8.5±3.2</td>
<td>7.7±2.5</td>
<td>11.8±3.6</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>71.0±8.0</td>
<td>6.6±3.9**</td>
<td>23.5±5.7</td>
<td>9.3±4.2</td>
<td>24.2±9.5</td>
<td>5.8±1.1</td>
</tr>
<tr>
<td><strong>ADP (20µM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>58.0±8.4</td>
<td>61.0±11.5</td>
<td>41.2±8.3</td>
<td>31.2±9.2</td>
<td>54.0±9.0</td>
<td>53.6±10.1</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>52.8±7.3</td>
<td>19.8±13.7*</td>
<td>36.3±11.3</td>
<td>3.6±2.9*</td>
<td>33.3±9.2</td>
<td>13.8±10.9</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>41.0±10.0</td>
<td>0.4±0.4**</td>
<td>28.2±10.4</td>
<td>3.0±1.3*</td>
<td>32.2±7.1</td>
<td>0.5±0.5*</td>
</tr>
<tr>
<td><strong>ADP (5 µM)</strong></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>47.2±6.0</td>
<td>58.0±11.1</td>
<td>22.2±6.1</td>
<td>21.0±7.0</td>
<td>32.4±10.0</td>
<td>39.4±12.7</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>32.3±6.1</td>
<td>18.2±13.8*</td>
<td>18.0±5.4</td>
<td>1.8±1.1**</td>
<td>16.0±5.4</td>
<td>11.2±6.9</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>23.8±6.2</td>
<td>1.2±0.6*</td>
<td>27.0±11.8</td>
<td>4.8±3.5*</td>
<td>12.3±3.8</td>
<td>0.5±0.5*</td>
</tr>
<tr>
<td><strong>γ-TB (25 nM)</strong></td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>58.7±10.3</td>
<td>55.5±10.5</td>
<td>42.0±8.1</td>
<td>44.8±12.0</td>
<td>61.0±11.9</td>
<td>56.0±13.9</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>58.3±8.6</td>
<td>12.3±5.7**</td>
<td>42.2±11.9</td>
<td>3.0±2.3**</td>
<td>27.3±11.7*</td>
<td>16.0±11.0**</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>61.0±5.9</td>
<td>4.2±1.7**</td>
<td>53.0±13.5</td>
<td>4.0±1.0**</td>
<td>10.2±1.9**</td>
<td>0.8±0.5**</td>
</tr>
</tbody>
</table>

*Platelet rich and platelet poor plasma were prepared from whole blood anticoagulated with 3.7% sodium citrate. Groups 0-5 are defined in Table 1.*
Data are expressed as mean±SE. When the values from different time points compared with baseline values within each group, * indicates p<0.05; ** indicates p<0.01. When the baseline values from each groups are compared with baseline value from control group, # indicates p<0.05; ## indicates p<0.01.
Table 3

*Ex vivo* Platelet Aggregation Responses to Arachidonic Acid (AA, 0.65 mM), ADP (5 and 20 µM), and Gamma-Thrombin (γ-TB, 25 nM)*\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Group 0</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AA (0.65 mM)</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>57.8±2.2</td>
<td>53.2±6.4</td>
<td>32.8±12.3</td>
<td>42.5±7.4</td>
<td>54.0±15.3</td>
<td>50.2±11.2</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>73.7±9.2</td>
<td>33.2±16.5</td>
<td>54.8±12.7</td>
<td>22.8±6.7</td>
<td>48.0±7.0</td>
<td>14.2±3.2*</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>68.6±8.3</td>
<td>47.7±13.4</td>
<td>33.0±10.6</td>
<td>32.0±10.8</td>
<td>60.2±7.9</td>
<td>24.5±11.6</td>
</tr>
<tr>
<td><strong>ADP (20µM)</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>51.0±4.3</td>
<td>47.7±7.8</td>
<td>37.3±11.6</td>
<td>50.8±9.1</td>
<td>55.8±16.3</td>
<td>48.4±10.5</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>50.7±9.4</td>
<td>42.3±16.5</td>
<td>55.8±15.3</td>
<td>32.8±8.1</td>
<td>62.2±10.3</td>
<td>13.3±5.2*</td>
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<tr>
<td>4hr-tPA</td>
<td>65.2±10.5</td>
<td>55.2±14.0</td>
<td>43.0±13.3</td>
<td>18.3±3.9*</td>
<td>60.5±10.6</td>
<td>6.0±3.1**</td>
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<td><strong>ADP (5 µM)</strong></td>
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<tr>
<td>Baseline</td>
<td>50.5±3.5</td>
<td>39.3±9.1</td>
<td>43.0±14.1</td>
<td>47.5±9.3</td>
<td>47.3±14.2</td>
<td>44.0±11.1</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>55.2±11.1</td>
<td>37.0±15.0</td>
<td>49.5±17.6</td>
<td>23.4±8.0</td>
<td>54.5±10.8</td>
<td>18.7±11.2</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>68.6±5.6</td>
<td>41.8±15.4</td>
<td>28.3±12.0</td>
<td>16.2±2.7*</td>
<td>58.0±10.5</td>
<td>3.8±1.1*</td>
</tr>
<tr>
<td><strong>γ-TB (25 nM)</strong></td>
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</tr>
<tr>
<td>Baseline</td>
<td>51.7±4.7</td>
<td>46.7±5.9</td>
<td>26.5±9.9</td>
<td>31.2±6.9</td>
<td>22.0±4.0</td>
<td>37.2±8.5</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>59.3±6.8</td>
<td>29.0±13.9</td>
<td>35.0±13.2</td>
<td>17.0±5.6</td>
<td>27.7±11.2</td>
<td>18.7±5.1</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>62.2±8.6</td>
<td>28.5±7.5</td>
<td>23.3±6.3</td>
<td>16.8±9.2</td>
<td>30.8±8.3</td>
<td>10.0±5.1</td>
</tr>
</tbody>
</table>
Platelet rich and platelet poor plasma were prepared from whole blood anticoagulated with heparin, 5 IU/ml. Groups 0-5 are defined in Table 1. Data are expressed as mean±SE. When the values from different time points are compared with baseline values within each group, * indicates p<0.05; ** indicates p<0.01.

Table 4.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Pre-adjunctive</th>
<th>Pre-tPA</th>
<th>1hr post-tPA</th>
<th>3hr post-tPA</th>
<th>4hr post-tPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BT (sec)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>140.5±15.4</td>
<td>121.0±16.1</td>
<td>256.2±34.6</td>
<td>256.0±31.7</td>
<td>245.7±33.5</td>
<td>173.9±19.8</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>117.3±14.6</td>
<td>175.5±32.6</td>
<td>189.8±16.1</td>
<td>330.7±48.3</td>
<td>242.2±43.1</td>
<td>305.0±61.0</td>
</tr>
<tr>
<td>4hr-tPA</td>
<td>95.0±22.8</td>
<td>154.3±20.5</td>
<td>211.7±12.2</td>
<td>252.5±73.9</td>
<td>257.4±21.9</td>
<td>620.0±98.7**</td>
</tr>
</tbody>
</table>

| **aPTT (sec)**      |          |                |         |              |              |              |
| Baseline            | 120.2±5.5 | 118.8±9.5      | 128.2±5.3 | 129.5±6.3    | 123.0±4.0    | 123.9±6.6    |
| Pre-tPA             | 108.0±7.5 | 101.3±7.8      | 123.0±9.4 | 128.0±7.1    | 116.2±6.2    | 116.6±6.2    |
| 4hr-tPA             | 102.6±5.8 | 105.5±8.5      | 124.0±5.9 | 111.6±3.2    | 146.7±6.8    | 133.0±9.6    |

Data are expressed as mean±SE. Groups 0-5 are defined in Table 1. When the values at different time points are compared with the baseline values within each group, * indicates p<0.05; ** indicates p<0.01.
Table 5

Incidence of Left Circumflex Coronary Artery Reocclusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Adjunctive Treatment</th>
<th>Incidence of Reocclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 0</td>
<td>Control Group</td>
<td>6/6</td>
</tr>
<tr>
<td>Group 1</td>
<td>CRL42796</td>
<td>3/6</td>
</tr>
<tr>
<td>Group 2</td>
<td>Aspirin</td>
<td>5/6</td>
</tr>
<tr>
<td>Group 3</td>
<td>Aspirin+CRL42796</td>
<td>2/6</td>
</tr>
<tr>
<td>Group 4</td>
<td>Aspirin+Enoxaparin</td>
<td>2/6</td>
</tr>
<tr>
<td>Group 5</td>
<td>Aspirin+Enoxaparin+CRL42796</td>
<td>0/5</td>
</tr>
</tbody>
</table>
Blood sample for aPTT, complete blood counts, and *ex vivo* platelet aggregation using citrate or heparin anticoagulated blood for preparation of PRP.

**Experimental Protocol**

- 20 min: Anodal Current ON
- 40-60 min: Occlusive Thrombus
- 30 min: Anodal Current OFF
- 30 min: Start Adjunctive Treatment
- 15 min: Allow Thrombus to Age
- 60 min: Start rt-PA Loading Dose Plus Infusion
- 120 min: Monitor
- 60 min: Stop Adjunctive Treatment
Figure 3

![Graph showing Total Reperfusion Time (min) for different groups (G0 to G5) with n values (n=6 for G0, G1, G2, G3, G4, G5).](image_url)

- G0: n=6
- G1: n=6
- G2: n=6
- G3: n=6
- G4: n=5
- G5: n=5

* and ** indicate statistical significance.
Figure 4

Anodal Current "ON"

Adjunctive Regimens

Flow Patency Score

Patency Score   60min-tPA  120min-tPA  180min-tPA  240min-tPA
Group 0        1.0 ± 0.3  0.0 ± 0.0  0.0 ± 0.0  0.0 ± 0.0
Group 1        1.7 ± 1.4  1.0 ± 0.6  1.2 ± 0.6  1.0 ± 0.6
Group 2        1.5 ± 0.6  1.0 ± 0.6  0.8 ± 0.5  0.5 ± 0.5
Group 3        2.2 ± 0.3  1.5 ± 0.6  1.5 ± 0.6  1.5 ± 0.6
Group 4        1.7 ± 0.3  1.0 ± 0.5  1.3 ± 0.6  1.3 ± 0.5
Group 5        3.0 ± 0.0** 3.0 ± 0.0** 3.0 ± 0.0** 3.0 ± 0.0**

rt-PA i.c.

Thrombosis