Antithrombotic Efficacy of Thrombin Inhibitor L-374,087: Intravenous Activity in a Primate Model of Venous Thrombus Extension and Oral Activity in a Canine Model of Primary Venous and Coronary Artery Thrombosis

Departments of Pharmacology (J.J.C., M.A.H., G.R.S., G.L.S., A.A.W., J.J.L), Biological Chemistry (S.J.G., T.R.H., J.A.K., J.A.S.), Laboratory Animal Resources (D.B.G.), and Medicinal Chemistry (D.L.D., P.E.J.S., J.P.V.), Merck Research Laboratories, West Point, Pennsylvania

Accepted for publication November 23, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

The small molecule direct thrombin inhibitor L-374,087 was characterized across species in an in vitro activated partial thromboplastin clotting time (aPTT) assay and in vivo in rhesus monkey and dog thrombosis models. In vitro in rhesus, dog, and human plasma, L-374,087 concentrations eliciting 2-fold increases in aPTT were 0.25, 1.9, and 0.28 μM, respectively. In anesthetized rhesus monkeys, 300 μg/kg bolus plus 12 μg/kg/min and 300 μg/kg bolus plus 30 μg/kg/min L-374,087 i.v. infusions significantly reduced jugular vein thrombus extension, with both regimens limiting venous thrombus extension to 2-fold that of baseline thrombus mass compared with a 5-fold extension observed in the vehicle control group. Antithrombotic efficacy in the rhesus with the lower-dose regimen was achieved with 2.3- to 2.4-fold increases in aPTT and prothrombin time. In a conscious instrumented dog model of electrolytic vessel injury, the oral administration of two 10 mg/kg L-374,087 doses 12 h apart significantly reduced jugular vein thrombus mass, reduced the incidence of and delayed time to occlusive coronary artery thrombosis, and significantly reduced coronary artery thrombus mass and ensuing posterolateral myocardial infarct size. Antithrombotic efficacy in the dog was achieved with 1.6- to 2.0-fold increases in aPTT at 1 to 6 h after oral dosing with L-374,087. These results indicate significant antithrombotic efficacy against both venous and coronary arterial thrombosis with L-374,087 with only moderate elevations in aPTT or prothrombin time. The oral efficacy of L-374,087 characterizes this compound as a prototype for the further development of orally active direct thrombin inhibitors.

Conventional antithrombotic therapies possess significant mechanistic and therapeutic limitations (Weitz, 1996). The serine protease thrombin, due to its key position and multifactorial roles in coagulation and platelet aggregation, has emerged as a prominent target in the search for novel, effective antithrombotic agents (FitzGerald, 1996; Weitz, 1996). Specifically, effort has been directed toward the synthesis of potent and selective small molecule direct inhibitors of thrombin with appropriate physicochemical and pharmacokinetic properties permitting oral dosing (Ripka and Vlasuk, 1997). The identification of a small molecule thrombin inhibitor with sufficient bioavailability to permit low-frequency oral dosing would significantly expand the use of such an agent beyond acute treatment to chronic prophylaxis and the management of vaso-occlusive disorders.

Chemistry strategies used by this group to design potent and selective small molecule direct thrombin inhibitors have been reported recently. Starting with the well known tripeptide D-Phe-Pro-Arg-H motif, the replacement of arginine with a trans-aminocyclohexylglycine ketoamide residue in the P1 position led to the highly potent transition-state inhibitor L-370,518 (Brady et al., 1995). Removal of the electrophilic ketoamide functionality of L-370,518 led in turn to the noncovalent inhibitor L-371,912 (Lyle et al., 1997). Subsequent efforts to modulate lipophilicity through the introduction of novel P3 groups (Tucker et al., 1997; Brady et al., 1998), the incorporation of a central aminopyridinone P2 group (Sanderson et al., 1997), and the introduction of a less basic and less polar aminopyridyl P1 group (Feng et al., 1997) to improve oral bioavailability have culminated in the synthesis of L-374,087 (Fig. 1) (Sanderson et al., 1998). L-374,087 is a potent (Kᵢ = 0.5 nM) and selective direct inhibitor of thrombin with demonstrated antithrombotic efficacy in a rat model.
of FeCl₃-induced carotid artery thrombosis and with oral bioavailabilities of 19% and 44% in monkeys and dogs, respectively (Sanderson et al., 1998). In the present study, the anticoagulant potency of L-374,087 was compared among dog, rhesus monkey, and humans through the determination of effects on in vitro activated partial thromboplastin clotting time (aPTT) to provide a perspective on species selection for experimental animal models. The in vivo antithrombotic efficacy of L-374,087 was then characterized after intravenous administration in an anesthetized rhesus monkey model of jugular vein thrombus extension and after oral administration in a conscious chronically instrumented dog model of electrolytic injury-induced jugular vein and left circumflex artery (LCx) thrombosis.

Materials and Methods

In Vitro aPTT Assay

The aPTT was measured with an MLA Electra 900 Automatic Coagulation Timer (Medical Laboratory Automation, Inc.). The aPTT values were determined with citrated plasma from dog, rhesus monkey, or human in the absence or presence of increasing concentrations of exogenously added L-374,087. The values reported are the inhibitor concentrations (in the final assay volume) that raise the clotting time by 2-fold.

In Vivo Thrombosis Models

All procedures related to the use of animals in these studies were reviewed and approved by the Institutional Animal Care and Use Committee at Merck Research Laboratories at West Point and conform with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

Anesthetized Rhesus Monkey Jugular Vein Thrombus Extension Model

Surgical Preparation. Rhesus monkeys (macaca mulatta) of either sex (3.3–8.7 kg) were sedated with ketamine HCl (10 mg/kg i.m.), anesthetized with sodium pentobarbital (12.5 mg/kg i.v.), intubated, and ventilated with room air (20 ml/kg, 12 breaths/min) using a positive pressure ventilator (Harvard Apparatus, South Natick, MA). The right femoral artery and the right and left femoral veins were cannulated for blood collection and the continuous monitoring of hemodynamic parameters (Statham P231D; Gould Inc., Cleveland, OH; 7E polygraph; Grass Instrument Division, West Warwick, RI), supplemental anesthesia infusion and compound administration, respectively. The left and right internal jugular veins were exposed between the clavicle and mandible by blunt dissection and instrumented cephally with Doppler flow probes (CBI-8000; Crystal Biotech, Hopkinton, MA) for continuous monitoring of blood flow velocity.

Treatment Groups and Experimental Protocol. After the surgical preparation of both jugular veins, consistent, standardized external vascular constrictions were applied by placing a 29-gauge hypodermic needle parallel to each vessel, tying a silk suture around both vessel and needle, and then removing the needle. Endothelial damage was created by external compression, blood flow through these vessels was interrupted with microvascular clips, and thrombus formation was initiated by the injection of thromboplastin (100 µl per vessel, lyophilized rabbit brain thromboplastin; Sigma Chemical Co., St. Louis, MO) directly into the occluded vessel segments. Venous stasis was maintained for 30 min in both vessels to allow thrombus formation. After this 30-min period, the thrombus in one jugular vein was removed, and baseline jugular vein wet thrombus mass was determined. Also at this time, the external occlusion used to interrupt blood flow to the remaining jugular vein segment was removed. The remaining thrombus was allowed to develop for an additional 300 min. At 330 min after the initiation of thrombus formation, this remaining “treatment” thrombus was removed and weighed. The within-animal difference between the thrombus masses determined from the two jugular veins (330-min mass minus baseline 30-min mass) was considered the amount of thrombus extension. A pilot study with untreated monkeys (n = 3) confirmed that baseline 30-min thrombus masses were similar in right and left jugular veins (right, 26.7 ± 7.7 mg; left, 30.9 ± 13.3 mg).

Eleven rhesus monkeys were randomly assigned to one of three i.v. treatment groups: vehicle control (20% PEG-200 in saline, bolus plus infusion (n = 4), 300 µg/kg bolus plus 12 µg/kg/min L-374,087 (n = 4), or 300 µg/kg bolus plus 30 µg/kg/min L-374,087 (n = 3). Treatments were initiated 5 min after the restoration of blood flow to occluded jugular vein segments (i.e., after 30 min of venous stasis and the removal of one segment for the determination of baseline 30-min thrombus mass), and infusions were continued for 300 min. Blood samples were drawn, and forearm template bleeding times were determined before the 30 min of stasis and at 30, 60, 120, 180, 240, and 300 min after the start of treatment. Blood samples were used either as whole blood or for plasma preparation for the ex vivo measurements of coagulation assays [aPTT, prothrombin time (PT), and activated clotting time (ACT)], whole blood platelet count, hemoglobin, hematocrit, and plasma L-374,087 levels (described below). Thrombin clotting time (TT) also was determined (described below) as an immediate bioassay of effective plasma thrombin inhibitor concentration. ACT values were measured only in the high-dose L-374,087 treatment group. Mean arterial pressure and lead II electrocardiogram were monitored continuously.

Conscious Canine Model of Jugular Vein and LCx Thrombosis

Surgical Preparation. Purpose-bred mongrel dogs of either sex (8–11 kg) were instrumented during general anesthesia induced by isoflurane. After a left thoracotomy at the fifth intercostal space, the proximal LCx was isolated, and a stimulation electrode composed of 30-gauge silver-plated copper wire terminating with the tip of a 25-gauge stainless steel hypodermic needle was inserted through the wall and into the lumen of the vessel. The LCx stimulation electrode was secured by suture to the surface of the heart. The left jugular vein also was isolated, and a stimulation electrode identical to that described above was inserted into the vein and anchored with sutures to surrounding muscle. Four 10-mm-diameter silver disk electrodes were implanted s.c. for use as limb electrodes for electrocardiographic monitoring and to serve as the ground for the jugular vein and LCx stimulation electrodes. Surgical incisions were closed, and the animals were allowed to recover from anesthesia.

Treatment Groups and Experimental Protocol. At 2 days after surgical preparation, conscious dogs were randomized to an untreated control group (total n = 14 entered) or an L-374,087 treatment group (total n = 8 entered) in which L-374,087 was administered as two oral doses of 10 mg/kg by gastric lavage in 1% methylcellulose, with the first dose administered 1 h before the simultaneous initiation of venous and arterial electrolytic injury and the second dose administered 12 h after the first dose (i.e., every 12 h). Technical failures in this model, including premature occlusion...
sion of the LCx secondary to surgical instrumentation detected prospectively by the presence of ventricular ectopy as well as incorrect jugular vein and/or LCx stimulation electrode placement determined retrospectively by postmortem visual inspection, reduced the total number for the untreated control group to 14 jugular vein and 9 LCx determinations and that for the L-374,087 treatment group to 7 jugular vein and 5 LCx determinations.

In both groups, LCx injury was produced by the application of 50 μA of anodal current to the vessel for a period of 3 h, whereas jugular vein injury was produced by the application of 35 μA of anodal current to the vessel for a period of 2 h. After 3 h of LCx electrical stimulation, the dogs were equipped with Holter monitors for continuous electrocardiographic monitoring. In the L-374,087 treatment group, blood samples for determination of aPTT and plasma [L-374,087] (described below) were drawn before treatment (baseline), at 1 h after the first oral dose (i.e., time of simultaneous initiation of jugular vein and LCx electrolytic injury), at 4 h after the first oral dose (i.e., time of termination of LCx electrolytic injury, 1 h after the termination of jugular vein electrolytic injury), at 6 h after the first oral dose, and at 24 h after the first oral dose (i.e., 12 h after the second oral dose, representing a trough time point with every-12-h dosing). TT also was determined (described below) as an immediate bioassay of effective plasma thrombin inhibitor concentration. At 24 h after the simultaneous initiation of jugular vein and LCx electrical stimulation, dogs were euthanized by anesthetic agent overdose, the jugular vein and LCx were dissected and inspected, and wet thrombus at the site of electrical stimulation was retrieved and weighed. Posterolateral myocardial infarct size was determined by slicing the heart into 1-cm-thick transverse sections that were incubated in 0.4% triphenyltetrazolium chloride solution. The precipitate, whereas infarcted tissue remains pale. Infarct size was quantified gravimetrically and expressed as a percentage of the left ventricle. Holter monitor tapes were analyzed, and the first electrocardiographic manifestation of myocardial ischemia (ST-segment change and/or ventricular ectopy), expressed as time after initiation of LCx electrical stimulation, was taken to represent the time of thrombotic occlusion of the LCx.

Hematological Assays

Measurement of Ex Vivo Coagulation Responses, Whole Blood Platelet Counts, Hematocrit, and Hemoglobin. Effects on the intrinsic (aPTT) in plasma prepared from blood taken with sodium citrate, final concentration 0.38%, and ACT in nonanticoagulated whole blood and extrinsic (PT in plasma prepared from blood taken with sodium citrate) coagulation pathways were determined using standard reagents and an MLA Electra 900C for plasma assays (aPTT and PT) and a Medtronic ACT II timer for whole blood measurements (ACT). Whole blood platelet counts, hematocrit, and hemoglobin levels were determined using an automated hematology analyzer (Biochem ImmunoSystems).

TT. TT was determined in the present study using standard reagents and an MLA Electra 900C and used as an immediate bioassay of effective plasma thrombin inhibitor concentration. Subsequent enzymatic and HPLC assays (below) then were used to determine actual plasma L-374,087 concentration.

Forearm Template Bleeding Time. Template bleeding times were measured in anesthetized rhesus monkeys with a Simplate bleeding time device (Organon Teknika). A blood pressure cuff was placed on the upper arm and inflated to 50 mm Hg; uniform incisions were made on the muscular part of the forearm, and the duration of bleeding was timed to a maximum of 20 min.

Determination of Plasma [L-374,087]. The plasma concentrations of L-374,087 were determined initially by enzymatic assay in the rhesus monkey thrombosis studies and subsequently by HPLC in the canine thrombosis studies when the latter assay became available. The two assays were appropriately cross-validated. For the enzymatic assay, 0.2 ml of platelet-poor plasma was mixed with 2 ml of acetonitrile and centrifuged (2500 rpm for 10 min). The supernatant was dried with nitrogen gas using a Reacti-Therm III Heating Module (Pierce, Rockford, IL). The sample was resuspended in 0.4 ml of HBSP (50 mM HEPES, 150 mM NaCl, 0.1% PEG-8000, pH 7.4). Then, 5 to 50 μl of resuspension (diluted appropriately in HBSP buffer) was mixed with 50 μl of 30 nM human thrombin (Enzyme Research Labs, South Bend, IN); the total volume brought up to 100 μl with HBSP buffer. The reaction was incubated for 10 min at room temperature. Then, 10 μl of 2.5 mM Sar-Pro-Arg-pna (Sigma) was added, and substrate hydrolysis was monitored at 405 nm using a Thermo-max microplate reader (Molecular Devices, Menlo Park, CA). The v/v ratios were calculated, and the plasma inhibitor concentrations were deduced from a standard curve constructed in HBSP buffer spiked with known concentrations of inhibitor. Concentrations were corrected for percent recovery of platelet-poor plasma spikes of known inhibitor concentrations treated as described above.

For the HPLC assay, plasma samples were prepared as above and reconstituted in 250 μl of buffer A (90% 4.5 mM heptane sulfonic acid in 0.006 N HCl/10% acetonitrile). Samples (200 μl) were analyzed by HPLC (Waters) using a polymeric reverse-phase column (Poros R1 column 0.21 × 10 cm; Perceptive Biosystems). L-374,087 was eluted at 5.8 min with an isocratic gradient using 80% buffer A and 20% buffer B (90% acetonitrile/10% water) at a flow rate of 1 ml/min. L-374,087 was detected by monitoring for its intrinsic fluorescence (excitation, 320 nm; emission, 375 nm).

Statistical Analysis

Data are expressed as mean ± S.E.M. Comparisons between two treatment groups were performed using a two-tailed, unpaired Student’s t test. Comparisons among more than two groups were performed with ANOVA, followed by a Fisher’s protected least significant difference test of all pairwise comparisons. Differences at the level of p < .05 were considered significant.

Results

In Vitro aPTT Assay

The inherent anticoagulant potency of L-374,087 among species was assessed through determination of the effect on in vitro aPTT, a measure of plasma coagulation triggered via the intrinsic pathway. Concentrations of L-374,087 in the final assay volume that increased aPTT 2-fold for various species were 0.25 μM for rhesus monkey (mean of three replicates), 1.9 μM for dog (mean of three replicates), and 0.28 μM for humans (mean of two replicates).

Anesthetized Rhesus Monkey Jugular Vein Thrombus Extension Model

Jugular Vein Thrombus Extension. Table 1 summarizes baseline 30-min jugular vein thrombus masses, 330-min treatment jugular vein thrombus masses, and within-animal thrombus extensions (330-min minus baseline 30-min jugular vein thrombus masses) induced by venous stasis in anesthetized rhesus monkeys in the vehicle control and 300 μg/kg plus 12 μg/kg/min and 300 μg/kg plus 30 μg/kg/min L-374,087 i.v. treatment groups. Baseline 30-min thrombus masses were somewhat variable but did not differ statistically among treatment groups. In the vehicle control group, thrombus extension resulted in a 330-min treatment thrombus mass that was approximately 5-fold greater than the baseline 30-min thrombus mass. In the lower- and higher-dose L-374,087 treatment groups, both the 330-min jugular vein thrombus masses and the thrombus extensions were reduced significantly (p < .01) compared with vehicle control.
Steady-state increases in clotting times observed with the on all clotting times evaluated were consistently observed. Dose-related effects peaked elevations in BT in both L-374,087 treatment groups statistically greater than that of the vehicle control group, though BT in the lower-dose L-374,087 treatment group was approximately 89 s. Steady-state plasma L-374,087 concentrations at 30 to 300 min of infusion in the two i.v. L-374,087 treatments, respectively, were aPTT, 2.4- and 5.1-fold; PT, 2.3- and 5.5-fold; and TT, which served as a bioassay for effective thrombin inhibitor concentration, 7.3- and 25.0-fold. ACT, determined in the higher-dose L-374,087 treatment group, was increased 3.5-fold (baseline ACT, approximately 89 s). Steady-state increases in clotting times observed with the 300 μg/kg plus 12 μg/kg/min and 300 μg/kg plus 30 μg/kg/min L-374,087 i.v. treatments, respectively, were aPTT, 2.4- and 3.5-fold; PT, 2.3- and 5.5-fold; and TT, which served as a bioassay for effective thrombin inhibitor concentration, 7.3- and 25.0-fold. ACT, determined in the higher-dose L-374,087 treatment group, was increased 3.5-fold (baseline ACT, approximately 89 s). Steady-state plasma L-374,087 concentrations at 30 to 300 min of infusion in the two i.v. L-374,087 treatment groups also were dose related and consistent with observed increases in clotting times. Plasma L-374,087 concentrations, expressed as steady-state mean and range across individual time points during 30 to 300 min of infusion, respectively, were 300 μg/kg plus 12 μg/kg/min, 1.20 ± 0.02 and 0.86 to 1.63 μM; and 300 μg/kg plus 30 μg/kg/min, 2.84 ± 0.11 and 1.32 to 3.36 μM.

**Bleeding Time, Coagulation Times, and Plasma Inhibitor Concentrations.** Summarized in Table 2 are the mean (30- to 300-min infusion) values for changes in forearm template bleeding time (BT), aPTT, PT, and TT, as well as plasma L-374,087 concentrations at 30 to 300 min during vehicle, 300 μg/kg plus 12 μg/kg/min, and 300 μg/kg plus 30 μg/kg/min L-374,087 i.v. infusion. Although BT in the lower-dose L-374,087 treatment group was statistically greater than that of the vehicle control group, peak elevations in BT in both L-374,087 treatment groups were modest (1.1- to 1.5-fold increases). Dose-related effects on all clotting times evaluated were consistently observed. Steady-state increases in clotting times observed with the 300 μg/kg plus 12 μg/kg/min and 300 μg/kg plus 30 μg/kg/min L-374,087 i.v. treatments, respectively, were aPTT, 2.4- and 3.5-fold; PT, 2.3- and 5.5-fold; and TT, which served as a bioassay for effective thrombin inhibitor concentration, 7.3- and 25.0-fold. ACT, determined in the higher-dose L-374,087 treatment group, was increased 3.5-fold (baseline ACT, approximately 89 s). Steady-state plasma L-374,087 concentrations at 30 to 300 min of infusion in the two i.v. L-374,087 treatment groups also were dose related and consistent with observed increases in clotting times. Plasma L-374,087 concentrations, expressed as steady-state mean and range across individual time points during 30 to 300 min of infusion, respectively, were 300 μg/kg plus 12 μg/kg/min, 1.20 ± 0.02 and 0.86 to 1.63 μM; and 300 μg/kg plus 30 μg/kg/min, 2.84 ± 0.11 and 1.32 to 3.36 μM.

**Conscious Canine Model of Jugular Vein and LCx Thrombosis.** Table 3 summarizes the effects of the oral administration of 10 mg/kg L-374,087, two doses 12 h apart (i.e., every 12 h), compared with untreated controls on jugular vein and LCx thrombosis induced by electrolytic injury in conscious dogs. In the untreated control group, the 24-h jugular vein thrombus mass, 34.9 ± 6.8 mg, was large and somewhat variable. Oral treatment with L-374,087 resulted in a significant (p < .05), approximately 60% reduction in jugular vein thrombus mass. Similarly, oral administration of L-374,087 completely prevented the development of occlusive LCx coronary artery thrombosis in two of five animals tested, significantly (p < .01) delayed the onset of posterolateral myocardial ischemia in the remaining animals that developed occlusive LCx thrombosis, and significantly (p < .01) reduced both 24-h LCx thrombus mass and ensuing infarct size in the entire treatment group.

**Coagulation Times and Plasma Inhibitor Concentrations.** Summarized in Table 4 are the values for changes in aPTT and TT, as well as plasma L-374,087 concentrations at 1, 4, and 6 h after the first oral dose of 10 mg/kg L-374,087 and corresponding values at 24 h after the first oral dose (i.e., 12 h after the second 10-mg/kg oral dose). Peak increases in aPTT and TT, the latter again serving as a bioassay for effective thrombin inhibitor concentration, were 2.0- and 5.1-fold occurring at the 1-h time point. In the 1- to 6-h postdose time frame, aPTT was maintained at 1.6- to 2.0-fold in-

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle Control (n = 4)</th>
<th>L-374,087 300 μg/kg + 12 μg/kg/min (n = 4)</th>
<th>L-374,087 300 μg/kg + 30 μg/kg/min (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-min baseline jugular vein thrombus mass (mg)</td>
<td>15.2 ± 1.1</td>
<td>23.2 ± 6.7</td>
<td>9.6 ± 1.9</td>
</tr>
<tr>
<td>330-min treatment jugular vein thrombus mass (mg)</td>
<td>80.9 ± 7.4</td>
<td>40.5 ± 5.0*</td>
<td>21.0 ± 6.6*</td>
</tr>
<tr>
<td>Thrombus extension (mg/μl)</td>
<td>65.7 ± 7.8</td>
<td>17.4 ± 4.3*</td>
<td>11.4 ± 4.9*</td>
</tr>
</tbody>
</table>

*Within-animal difference between 330-min treatment and 30-min baseline jugular vein thrombus masses.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>BT (μM)</th>
<th>aPTT (μM)</th>
<th>PT (μM)</th>
<th>TT (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control (n = 4)</td>
<td>1.11 ± 0.02</td>
<td>1.00 ± 0.01</td>
<td>1.02 ± 0.01</td>
<td>1.01 ± 0.01</td>
</tr>
<tr>
<td>L-374,087: 300 μg/kg + 12 μg/kg/min (n = 4)</td>
<td>1.46 ± 0.03*</td>
<td>2.36 ± 0.05*</td>
<td>2.29 ± 0.02*</td>
<td>2.78 ± 0.05*</td>
</tr>
<tr>
<td>L-374,087: 300 μg/kg + 30 μg/kg/min (n = 3)</td>
<td>1.13 ± 0.03</td>
<td>3.47 ± 0.09*</td>
<td>5.49 ± 0.15*</td>
<td>24.96 ± 1.30*</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M.

a p < .05; b p < .01 compared with vehicle control value. c p < .05 compared with L-374,087, 300 μg/kg + 12 μg/kg/min value. Steady state, mean ± S.E.M. of six time points (30, 60, 120, 180, 240, and 300 min) during treatment infusion.

Absolute baseline value ranges for three treatment groups: BT = 1.9 ± 0.1 to 2.8 ± 0.3 min; aPTT = 18.3 ± 0.8 to 20.4 ± 2.2 s; PT = 13.5 ± 1.3 to 16.3 ± 0.6 s; TT = 8.4 ± 0.3 to 10.2 ± 0.5 s.
creases, whereas TT was maintained at 2.4- to 5.1-fold increases. At the 24-h time point, representing the 12-h trough after the second oral dose, 1.4- and 1.6-fold increases in aPTT and TT, respectively, were observed. Plasma L-374,087 concentration peaked at 8.19 ± 1.18 μM at 1 h after the first oral dose, was maintained at 3.58 ± 0.42 μM at 6 h after the first oral dose, and was 1.83 ± 0.28 μM at the 24-h time point (i.e., 12-h trough after the second oral dose).

**Discussion**

Despite traditional antithrombotic therapy with aspirin and/or heparin in acute coronary syndromes, there remains a significant incidence of progression of unstable angina to myocardial infarction, failure or incomplete reperfusion as well as reoclusion after thrombolysis in acute myocardial infarction, and abrupt thrombotic closure after coronary angioplasty (Challapalli et al., 1996). Similarly, venous thromboembolism remains a major complication in patients undergoing high-risk surgical procedures (i.e., orthopedic surgery) despite the use of heparin, low molecular weight heparin, and oral coumarins (Agnelli and Sonaglia, 1997). Conventional antithrombotic agents possess mechanistic characteristics that underlie their clinical limitations. Standard unfractionated heparin is recognized as possessing multiple intrinsic limitations, including the absolute requirement for plasma antithrombin for anticoagulant activity; the inability of the heparin-antithrombin complex to access and inactivate clot-bound thrombin; inactivation by platelet factor IV and heparinase, which are released by activated platelets; avid binding to a variety of plasma proteins, including acute-phase reactants, a 0.3 to 2.4% incidence of heparin-induced thrombocytopenia, and restriction to parenteral administration (Hirsh, 1991a; Weitz, 1996). Many of these unfavorable characteristics contribute to variability in in vivo effec with unfractionated heparin, resulting in the need for routine laboratory monitoring. Relative to unfractionated heparin, low molecular weight heparins display an improved pharmacokinetic profile and predictable anticoagulant effects due to decreased binding to plasma proteins, thereby obviating the need for routine laboratory monitoring. However, low molecular weight heparins share with unfractionated heparin the absolute requirement for plasma antithrombin for anticoagulant activity, the inability to inactivate clot-bound thrombin, and restriction to parenteral administration (Weitz, 1997). Oral anticoagulant therapy is currently limited to coumarins such as warfarin. A major therapeutic limitation of oral coumarins is variability in patient response due to multiple dietary and drug interactions, again necessitating intensive laboratory monitoring. Furthermore, because the anticoagulant mechanism of coumarins involves inhibition of protein synthesis and protein turnover, multiple days of therapy are required before the achievement of effective anticoagulation (Hirsh, 1991b).

Thrombin plays a central role in thrombosis and coagulation. As the terminal enzyme in the coagulation cascade, thrombin converts circulating fibrinogen to insoluble fibrin monomers, which polymerize in clot formation. Thrombin also activates factor XIII, which cross-links fibrin and promotes clot stability; activates platelets leading to the secretion of granular contents and platelet aggregation, with the activated platelet surface providing a substrate for prothrombinase complex assembly; and stimulates its own production by activating factors V and VIII (FitzGerald, 1996; Weitz, 1996). Given its multiple and diverse procoagulant functions, extensive effort has been devoted to the development of potent, cofactor-independent direct inhibitors of thrombin as potential antithrombotic agents (Ripka and Vlasuk, 1997). The rationale for direct thrombin inhibition as an antithrombotic strategy has been supported in part by experience with...
hirudin, a naturally occurring 65-amino-acid protein isolated from the medicinal leech (*Hirudo medicinalis*) and its synthetic analog hirulog. Both hirudin and hirulog are parenterally administered antithrombin-independent inhibitors of thrombin that have access to and inhibit clot-bound thrombin (Maraganore and Adelman, 1996; Toschi et al., 1996). Clinical experience with hirudin and hirulog in varying disease states has been mixed. In patients with unstable angina or suspected acute myocardial infarction, the administration of hirudin for 3 days resulted in a significant reduction in cardiac events compared with unfractionated heparin at 7 days and 6 months after treatment, despite a "rebound" in the incidence of ischemic events at 1 to 5 days after the cessation of treatment (OASIS Investigators, 1997). However, the administration of hirudin or hirulog for 1 to 4 days to angioplasty patients failed to display superior long-term efficacy over heparin (Bittl et al., 1995; Serruys et al., 1995). In patients with acute myocardial infarction who are administered tPA or streptokinase, the adjunctive administration of initial higher-dose regimens of hirudin resulted in high incidence rates of major bleeding (Antman, 1994; GUSTO IIA Investigators, 1994; Neuhaus, 1994), whereas subsequent lower-dose regimens failed to show an efficacy advantage over heparin at 30 days after thrombolysis (Antman, 1996; GUSTO IIb, 1996). The adjunctive use of hirulog with streptokinase resulted in an improvement in vessel patency at 1 to 3 days compared with heparin; however, at 35 days after thrombolysis, there were no differences in clinical events between hirulog and heparin (White et al., 1997). Issues raised by the preceding studies regarding the parenteral use of these direct thrombin inhibitors in acute coronary syndromes include appropriate dose selection to achieve effective levels of anticoagulation; safety, particularly with regard to bleeding risk; and duration of therapy, with the results of several studies suggesting short-term benefit after acute treatment, followed by a longer-term reduction in efficacy. Hirulog and hirudin also have been investigated for the treatment of venous thrombosis. Both agents have been shown to reduce the incidence of deep vein thrombosis in orthopedic surgery patients, with hirudin reported to be superior to both heparin and low molecular weight heparin (Ginsberg et al., 1994; Eriksson et al., 1997a, b). Overall, clinical studies with hirudin and hirulog support potential therapeutic use for direct thrombin inhibitors in the treatment of vaso-occlusive disorders. However, the identification and development of an orally active agent would facilitate the assessment of the clinical usefulness of thrombin inhibition by permitting an extension of duration of therapy and an expansion to clinical targets amenable to chronic therapy.

L-374,087 is a potent ($K_I = 0.5$ nM) and rapid ($K_{inact} = 90$ $\mu$M$^{-1}$ s$^{-1}$) direct inhibitor of thrombin (Sanderson et al., 1998). In an initial characterization of antithrombotic efficacy, the continuous i.v. infusion of $10 \mu$g/kg/min L-374,087, achieving plasma concentrations of 588 $\pm$ 59 nM, was effective in preventing acute FeCl$_3$-induced occlusive carotid artery thrombosis in anesthetized rats (Sanderson et al., 1998). Concentrations of L-374,087 that elevated in vitro aPTT clotting times 2-fold in human and rat plasma were 210 and 250 nM, respectively, suggesting similar sensitivities to this agent in these two species. Pharmacokinetic studies with L-374,087 in dog and monkey indicated oral bioavailabilities of 44% and 19%, respectively (Sanderson et al., 1998). This report extends the pharmacological characterization of L-374,087 by demonstrating significant efficacy against both primary venous and arterial thrombosis, as well as venous thrombus extension with moderate effects on coagulation times in dog and primate. Furthermore, significant oral antithrombotic efficacy with this agent was demonstrated in the dog.

In the present study, the oral antithrombotic efficacy of L-374,087 was assessed in a conscious canine model in which thrombosis was elicited by electrolytic vessel injury. Electrolytic lesion-induced vessel injury is a widely used method in experimental thrombosis models, particularly for the production of coronary artery thrombosis in dog. Agents that have demonstrated efficacy when administered i.v. in acute, anesthetized dog models of electrolytic coronary artery thrombosis include the anti-GPIIb/IIIa antibody abciximab (ReoPro) (Mickelson et al., 1989), the small molecule GPIIb/IIIa antagonist tirofiban (Aggrastat; Merck, West Point, PA) (Lynch et al., 1995), and the thrombin inhibitor hirudin (Homeister et al., 1991). Electrolytic injury has been adapted for the production of jugular vein thrombosis in an acute anesthetized dog preparation (Rebello et al., 1997). Coronary artery electrolytic lesion also has been incorporated into conscious instrumented dog models for the assessment of oral antithrombotic agents (Cook et al., 1996; Cousins et al., 1996). In such an instrumented dog model, oral antithrombotic activity has been reported with the thrombin inhibitor CVS-1123, administered as three 20 mg/kg oral doses with each dose separated by a period of 4 h, resulting in peak approximately 10-fold increases in aPTT (Cousins et al., 1996).

The conscious canine model of coronary artery and jugular vein electrolytic injury described in the present study permits the simultaneous assessment of arterial and venous antithrombotic efficacy of an orally administered test agent. L-374,087, administered as two 10 mg/kg oral doses 12 h apart, significantly reduced jugular vein thrombus mass, reduced the incidence of and delayed time to occlusive coronary artery thrombosis, and significantly reduced coronary artery thrombus mass and ensuing postero lateral myocardial infarct size. Venous and arterial antithrombotic efficacies were achieved with a moderate 1.6- to 2.0-fold increase in aPTT at 1 to 6 h after oral dosing. aPTT remained elevated 1.4-fold at 12 h after the second oral dose of L-374,087, indicating prolonged in vivo anticoagulant activity. Plasma L-374,087 concentration at 1 to 6 h after oral dosing ranged between 3.5 and 8 $\mu$M and was 1.8 $\mu$M at 12 h after the second oral dose of L-374,087, suggesting a favorable pharmacokinetic profile for low-frequency oral dosing. However, based on in vitro aPTT values for L-374,087 indicating significantly lower sensitivity in dog versus human for this agent, plasma L-374,087 concentrations required for antithrombotic efficacy in dog likely overestimate those required in humans.

Primates have been used less extensively in the assessment of novel antithrombotic compounds. Agents that have demonstrated efficacy in primates include anti-GPIIb/IIIa antibodies in a cynomolgus monkey model of carotid artery platelet-dependent cyclic flow reductions (Coller et al., 1989) and the thrombin inhibitor hirudin in arteriovenous shunts in baboons (Kelly et al., 1991). Cook et al. (1995) reported hirudin, a cyclic peptide GPIIb/IIIa antagonist MK-852, and an antibody raised against the human thrombin receptor.
effective in abolishing platelet-dependent cyclic flow reductions of the carotid artery of African green monkeys. The rhesus monkey model described in the present study incorporates elements of venous stasis and preexisting thrombosis, features relevant to the clinical condition of deep vein thrombosis, to assess the ability of L-374,087 to inhibit thrombus growth. In both i.v. regimens tested, L-374,087 significantly reduced absolute jugular vein thrombus mass as well as thrombus extension, suggesting comparable efficacies between these two regimens and the potential for efficacy at an even lower dose. Antithrombotic efficacy in the rhesus model with the lower L-374,087 infusion regimen was achieved with moderate 2.3- to 2.4-fold increases in aPTT and PT and a steady-state plasma L-374,087 concentration of 1.2 µM. Based on in vitro aPTT values for L-374,087 indicating comparable sensitivities in rhesus versus humans, plasma L-374,087 concentrations required for antithrombotic efficacy in the rhesus preparation may represent a reasonable estimate of clinically relevant plasma concentration. As noted previously, the oral bioavailability of L-374,087 determined in monkey was lower than that of dog, 19% and 44%, respectively (Sanderson et al., 1998), such that an orally efficacious antithrombotic dose of L-374,087 in monkey would be absolutely lower than that in dog due to the increased sensitivity of primate to the inhibitor but at the same time would have to be sufficient to compensate for reduced bioavailability in primate relative to dog. BT, frequently used as an index of bleeding risk but known to lack strict correlation to the incidence of clinical bleeding events (Lind, 1991), was elevated only modestly with i.v. L-374,087 in the rhesus monkey. Ultimately, relationships among effects on clotting markers, antithrombotic efficacy, and bleeding risk with novel thrombin inhibitors such as L-374,087 must be established in clinical trials. The oral antithrombotic efficacy of L-374,087 in the present study characterizes this compound as a prototype for the further development and identification of orally active direct thrombin inhibitors.

References


Cook JJ, Sitko GR, Baskin EP, Lynch JJ, Lucas R, Shafer JA, Dan-


Send reprint requests to: Dr. Jacquelynn Cook, Merck Research Laboratories, WP46-300, West Point, PA 19486.