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Antithrombotic efficacy of RPR208566, a novel factor Xa inhibitor, in a rat model of carotid artery thrombosis

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Abstract

Coagulation factor Xa is the sole enzyme responsible for activating the zymogen prothrombin to thrombin, resulting in fibrin generation, platelet activation, and subsequent thrombus formation. Our objective was to evaluate the antithrombotic efficacy of the novel factor Xa inhibitor, 2-(3-carbamimidoyl-benzyl)-3-[(3′,4′dimethoxy-biphenyl-4-carbonyl)-amino]-butyric acid methyl ester-trifluoroacetate (RPR208566), in a well-established rat model of arterial thrombosis, and to compare the results with those obtained with argatroban and heparin, direct and indirect inhibitors of thrombin, respectively. Thrombus formation was initiated by placing a filter paper saturated with $FeCl_2$ on the adventia of the carotid artery for 10 min. Time-to-occlusion was measured from initiation of injury until blood flow reached zero. Formed thrombi were removed and weighed 60 min after the placement of the filter paper. RPR208566, heparin, and argatroban dose-dependently increased time-to-occlusion and reduced thrombus mass. When administered at 500 $\mu g/kg + 50$ $\mu g/kg/min$, RPR208566 prolonged time-to-occlusion to 56 ± 4 min (vs. 18 ± 2 min for vehicle) and reduced thrombus mass to 3.0 ± 0.7 mg (vs. 7.3 ± 0.6 mg for vehicle). The highest doses of argatroban (500 $\mu g/kg + 50$ $\mu g/kg/min$) and heparin (300 $U/kg + 10$ $U/kg/min$) increased time-to-occlusion to the maximum of 60 min and decreased thrombus mass to 5.5 ± 0.8 and 2.6 ± 0.3, respectively. The antithrombotic effects of heparin and argatroban at these doses were associated with increases in activated partial thromboplastin time of 5.6 ± 0.9- and 2.9 ± 0.3-fold over baseline, respectively. However, the highest dose of RPR208566 produced a modest 1.3 ± 0.1-fold increase in activated partial thromboplastin time. These results indicate that factor Xa inhibition with compounds such as RPR208566 may be an attractive mechanism for novel antithrombotic drug therapy.

Keywords: Antithrombotic; Coagulation factor Xa; Thrombin; Heparin; (In vivo)

1. Introduction

Heparin and low molecular weight heparins are currently established anticoagulant therapy for the treatment and prevention of thrombotic diseases. However, these agents have significant limitations such as their inability to inhibit catalytically-active thrombin and coagulation factor Xa that are incorporated in the thrombus (Hirsh and Levine, 1992). Consequently, more effective therapies need to be developed.

Recent focus has been on developing agents that directly inhibit thrombin (Tapparelli et al., 1993), thereby preventing coagulation and thrombin-induced platelet activation. For example, direct thrombin inhibitors such as hirudin and hirulog, which are derivatives of medicinal leech extracts, have been studied extensively, both preclinically and clinically (Callas and Fareed, 1995; Catella-Lawson, 1997). In addition, synthetic thrombin active-site inhibitors, such as argatroban and efegatran have also been studied extensively (Bush, 1992; Schwarz et al., 1997; Smith et al., 1997). In general, these agents have not provided the safety and efficacy advantages over heparin as originally hoped. For example, when administered at a dose that was considered safe, hirudin did not significantly reduce endpoints after arterial thrombosis (Antman and TIMI 9B Investigators, 1996; Topol and GUSTO IIb Investigators, 1996). Although results using argatroban (Jang et al., 1999) or hirulog (Theroux et al., 1995; White et al.,

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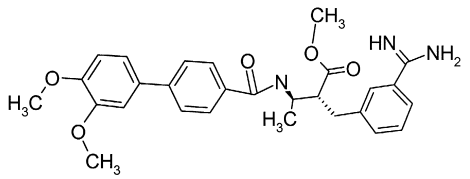

Fig. 1. Structure of RPR208566.

1997) as adjuncts to thrombolysis appear promising, further studies are required to conclusively determine the risk/benefit profile of these agents. It is not clear why direct thrombin inhibitors have not been more successful, but it is conceivable that the production of thrombin is not altered by these agents and that the coagulation cascade remains highly active, producing such a large amount of thrombin that is difficult to overcome by administration of safe doses of direct thrombin inhibitors. The recurrence of ischemic events following termination of thrombin inhibitor therapy (Theroux et al., 1992; Gold et al., 1993) is consistent with the theory that thrombin is continually generated, even during thrombin inhibitor treatment.

Consequently, recent efforts have been made to develop agents that prevent the production of thrombin earlier in the coagulation cascade. Coagulation factor Xa, because of its central position at the convergence point of both the ''contact'' and ''tissue factor/coagulation factor VIIa'' pathways of coagulation, plays a pivotal role in the activation of prothrombin (factor II) to yield thrombin (factor IIa), which ultimately results in the formation of fibrin and a stable clot. Therefore, a promising approach has been to develop agents that inhibit the serine protease Xa (thereby inhibiting thrombin production) without impairing platelet function, resulting in an improved efficacy to safety ratio (Herbert et al., 1996; Kawasaki et al., 1998).

The specific objective of this study was to evaluate the pharmacodynamics and antithrombotic efficacy of the novel factor Xa inhibitor, 2-(3-carbamimidoyl-benzyl)-3-[(3′,4′dimethoxy-biphenyl-4-carbonyl)-amino]-butyric acid methyl ester-trifluoroacetate (Fig. 1; RPR208566), and compare it to the thrombin active-site inhibitor, argatroban, and to the antithrombin-III dependent anticoagulant, heparin, in a well-established rat model of carotid artery thrombosis.

2. Materials and methods

2.1. Materials

RPR208566 was synthesized by the Medicinal Chemistry Section of the Department of Cardiovascular Drug Discovery at Rhône-Poulenc Rorer. Argatroban (Novastan) was obtained from Mitsubishi Chemical (Japan). Heparin Sodium (1000 USP units/ml) was obtained from Solopak Laboratories (Elk Grove Village, IL).

2.2. In vitro methods

Enzyme assays using chromogenic substrates were performed as follows. Human Xa and thrombin were obtained from Enzyme Research Laboratories, (South Bend, IN). Bovine trypsin was obtained from Sigma (St. Louis, MO). Plasmin was purchased from Diapharma Group (Franklin, OH). Tissue plasminogen activator (tPA, Activase) was obtained from Genentech (San Francisco, CA). The chromogenic substrates used were Spectrozyme Xa (American Diagnostica, Greenwich, CT), Pefachrome TH, Pefachrome tPA (Centerchem, Stamford, CT) and S-2765 and S-2366 (Diapharma Group, Franklin, OH) for Xa, thrombin, tPA, trypsin, and plasmin and aPC (activated Protein C), respectively.

All enzyme assays were performed at room temperature in 96-well microtiter plates with a final enzyme concentration of 1 nM. Compound dilutions were added to the wells containing buffer and enzyme and incubated for 30 min. The enzyme reactions were initiated by the addition of substrate and the color developed from the release of *p*-nitroanilide from each chromogenic substrate was monitored continuously for 5 min at 405 nm on a Thermomax microtiter plate reader (Molecular Devices, Sunnyvale, CA). IC_{50}s were determined using a four-parameter curve-fitting model (SoftMax Pro, Molecular Devices).

Activated partial thromboplastin time was measured with an MLA Electra 800 automatic coagulation timer (Orthodiagnostics, NJ). Citrated human (George King Biomedical, Overland Park, KS) and rat (Sprague–Dawley, Charles River) plasma were used in the assays. Freshly thawed plasma (100 μl) was mixed with 100 μl of compound dilutions followed by the automatic addition of 100 μl of actin activated cephaloplastin reagent (Dade, Miami, FL) and 100 μl of 0.035 M calcium chloride to start clot formation. Anticoagulant activity of RPR208566 was evaluated in order to determine the concentration required to double the plasma clotting time.

2.3. Experimental protocol for the rat thrombosis model

All procedures in this study were approved by the Rhône-Poulenc Rorer Animal Care and Use Committee, in compliance with the Animal Welfare Act Regulations and with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Experiments were performed on male Sprague–Dawley rats anesthetized with Inactin (100 mg/kg, i.p.). The trachea was intubated via a tracheotomy with polyethylene tubing (PE 205) to ensure airway patency. Catheters (PE50) were inserted into the right jugular vein for drug administration and into the right femoral artery for collection of blood samples. The right carotid artery was isolated and a piece of Parafilm ''M'' (American Can, Greenwich, CT) was placed under the vessel to separate it from the surrounding tissue. An electromagnetic flow probe (0.95 mm

Table 1
Potency and selectivity of RPR208566 against serine proteases using chromogenic, amidolytic assays

Enzyme	K_i (nM)	Selectivity (enzyme K_i / Xa K_i)
Factor Xa	1.31	1
Thrombin	> 3950	> 3000
Plasmin	890	680
Activated protein C	> 18,400	> 14,100
tissue Plasminogen Activator	> 8600	> 6600
Trypsin	190	140

lumen) was placed on the carotid artery and attached to a model 1401 flow meter (Skalar, Delft, Netherlands). Following baseline flow measurements of 10 min, vehicle or drug (RPR208566, argatroban, or heparin) was administered as an intravenous bolus plus a constant infusion for 75 min ($n = 5$ per treatment group). Saline was used as the vehicle for all drugs, and, because the experiments were performed over a discontiguous period, separate vehicle experiments were performed when each drug was tested to ensure that experimental conditions had not changed. After 15 min of drug infusion, vascular damage was produced by the local application of a piece of filter paper (2×5 mm), saturated with a 50% solution of $FeCl_2$, placed on top of the vessel downstream from the flow probe as described in the methods of Kurz et al. (1990), with minor modifications. The filter paper was removed after 10 min. The carotid artery was removed 60 min after the application of the filter paper whether or not occlusion had occurred. Time to occlusion was defined as the time from the application of $FeCl_2$ until blood flow decreased to zero. If the vessel did not occlude by 60 min, the time to occlusion was assigned a 60 min value for data analysis. The thrombus was removed and the wet weight was determined immediately on a Sartorius BP160P balance (Sartorius, Gottingen, Germany).

2.4. Coagulation and template bleeding time

Arterial blood samples (0.9 ml in 0.1 ml, 3.8% trisodium citrate) were collected prior to drug administration and at 15 and 75 min after initiation of drug administration. Blood samples were centrifuged to obtain platelet-poor plasma for 3 min at 10,000 rpm (4°C) in a Beckmann GS15R centrifuge (Beckmann Instruments, Palo Alto, CA). Prothrombin time and activated partial thromboplastin time were measured using a Microsample Coagulation Analyzer (MCA210, Bio Data, Horsham, PA) and Dade reagents (Thromboplastin-C and Actin FS Activated PTT reagent, Baxter Diagnostics, Deerfield, IL). Coagulation times greater than 200 s for activated partial thromboplastin time and 100 s for prothrombin time were assigned the maximum time for data analysis. To measure template bleeding time, uniform incisions were made on the toe pads with a Surgicutt (ITC, Edison, NJ) automated device. Blood was blotted at 30-s intervals using filter paper (Whatman, Maidstone, England) and the bleeding time was measured from the time of incision until blood no longer stained the filter paper.

2.5. Determination of plasma drug levels by ex vivo anti-Xa and anti- IIa activity

Platelet poor plasma was collected after centrifugation of each blood sample. Plasma samples were then frozen at −70°C until the assays for anti-Xa and anti-IIa activity were performed. Ex vivo anti-Xa activity of RPR208566 and heparin was determined using a chromogenic assay utilizing bovine Xa (1.2 nKat/ml) and Spectrozyme Xa (0.24 μM) prepared according to the manufacturer's instructions (American Diagnostica). A standard curve of RPR208566 or heparin was prepared using known concentrations of RPR208566 or heparin diluted in plasma. The plasma samples were thawed and either sample or standard

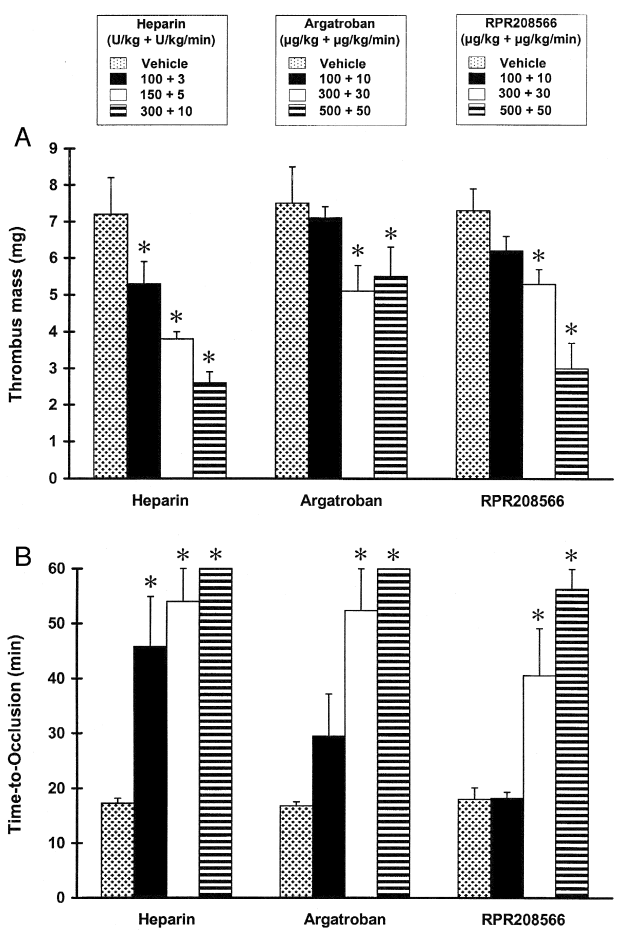

Fig. 2. Effect of RPR208566, argatroban, and heparin on thrombus mass (A) and time-to-occlusion (B) after intravenous administration in the rat carotid artery model of $FeCl_2$-induced thrombosis. $n = 5$ per treatment group. $^*P < 0.05$ vs. vehicle.

Table 2
Dosing regimen and plasma concentrations determined by ex vivo anti-Xa and anti-IIa activity

Heparin dose (U/kg + U/kg/min)	Plasma concentration 15 min (U/ml)	Plasma concentration 75 min (U/ml)
100 + 10	4.2 ± 0.3	5.1 ± 0.8
150 + 5	7.1 ± 1.1	11.2 ± 1.7
300 + 10	11.9 ± 2.6	13.8 ± 2.5
Argatroban dose (μg/kg + μg/kg/min)	**Plasma concentration 15 min (nM)**	**Plasma concentration 75 min (nM)**
100 + 10	180 ± 80	330 ± 110
300 + 30	1520 ± 410	2070 ± 500
500 + 50	2010 ± 360	1710 ± 540
RPR208566 dose (μg/kg + μg/kg/min)	**Plasma concentration 15 min (nM)**	**Plasma concentration 75 min (nM)**
100 + 10	210 ± 10	230 ± 30
300 + 30	760 ± 110	430 ± 90
500 + 50	980 ± 80	960 ± 130

Doses of heparin, argatroban, and RPR208566 during $FeCl_2$-induced thrombus formation in the anesthetized rat. Plasma concentrations were determined by ex vivo anti-Xa, IIa activity in chromogenic amidolytic assays, as described in Section 2. Values are mean ± S.E.M.

(20 μl) was added to a well on a 96-well microtiter plate. The assay buffer (60 μl), bovine Xa (60 μl) and Spectrozyme Xa (60 μl) were added at 1-min intervals to the wells of the plate, mixing between the addition of each reagent.

Anti-IIa activity of argatroban was determined by chromogenic assay utilizing bovine thrombin (0.28 U/ml), Spectrozyme TH (0.2 mM), and antithrombin-III prepared according to the manufacturer's instructions (American Diagnostica). A standard curve of argatroban was prepared using known concentrations of argatroban diluted in plasma. The plasma samples were thawed and either sample or standard (25 μl) was added to a well on a 96-well microtiter plate. The antithrombin-III (75 μl), bovine thrombin (50 μl) and Spectrozyme TH (50 μl) were added at 1-min intervals to the wells of the plate, mixing between the addition of each reagent.

All Xa (37°C) or IIa (25°C) plates were read kinetically at 405 nm using a SpectraMax 250, 96-well microplate spectrophotometer (Molecular Devices). Maximal velocity of the reaction in each well was obtained and analyzed using Softmax Pro software (Molecular Devices). The inhibition of Xa or IIa was determined for each standard or sample using the following equation: $(1 - V_{max}$ sample$/V_{max}$ control$) \times 100$. The standard curve was constructed as concentration of compound vs. percent inhibition of Xa or IIa. If the samples yielded percent inhibition of Xa or IIa outside of the linear portion of the standard curve (i.e., high plasma concentrations), the samples were further diluted with control plasma and re-assayed. Plasma concentration in each sample was interpolated from the standard curve and corrected for the dilution with plasma.

2.6. Statistics

The experiments were performed on groups of 5–6 rats each. All data are presented as the mean ± S.E.M. Data were analyzed by analysis of variance and multiple comparisons of means were performed using the least significant difference test. A *P*-value less than 0.05 was considered significant.

3. Results

3.1. In vitro potency and selectivity

RPR208566 is a potent inhibitor of Xa, with a K_i of 1.3 nM. In addition, RPR208566 demonstrated selectivity over other serine proteases involved in thrombosis and hemostasis (Table 1). For example, the K_is against thrombin, plasmin, and tPA were > 3950, 890, and > 8600, respectively. As a general measure of selectivity, RPR-208566 was tested against trypsin and was found to be

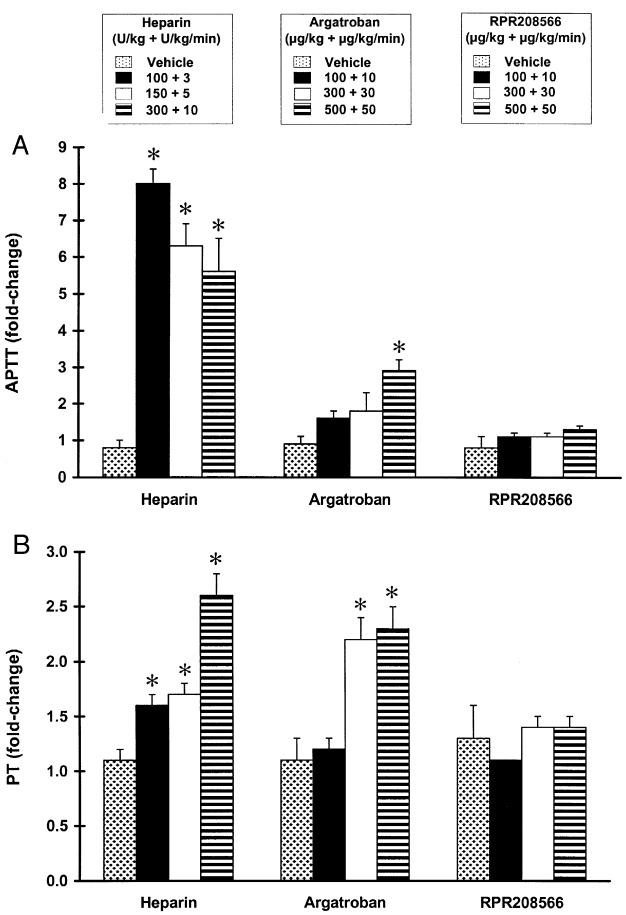

Fig. 3. Effect of RPR208566, argatroban, and heparin on activated partial thromboplastin time (APTT; panel A) and prothrombin time (PT; panel B) during $FeCl_2$-induced thrombosis in the rat carotid artery. Data are shown as fold-changes from pre-drug control measurements. $n = 5$ per treatment group. $^*P < 0.05$ vs. vehicle.

relatively less selective against trypsin (140-fold selective for Xa vs. trypsin).

The concentration of RPR208566 required to double activated partial thromboplastin time was 0.67 ± 0.01 μM in pooled human plasma ($n = 2$) and 1.53 ± 0.11 μM in pooled rat plasma ($n = 2$).

3.2. In vivo antithrombotic efficacy

Administration of RPR208566, heparin, or argatroban does dependently inhibited thrombus formation in the carotid artery (Fig. 2), as evidenced by decreases in thrombus mass and increases in time-to-occlusion. In the vehicle-treated animals, thrombus mass was 7.3 ± 0.1 mg and time-to-occlusion was 17 ± 1 min (Fig. 2). The highest dose of heparin, argatroban, and RPR208566 yielded thrombus masses of 2.6 ± 0.3, 5.5 ± 0.8, and 3.0 ± 0.7 mg, respectively. In addition, vessels remained patent for 60 min in all but one of the drug treated rats at the highest doses (the exception was one RPR208566-treated rat, which occluded at 42 min). The plasma concentrations of heparin, argatroban, and RPR208566 required to elicit this antithrombotic activity at the highest doses were approximately 12 IU/ml, 2000 nM, and 1000 nM, respectively (Table 2).

Activated partial thromboplastin time was increased > 5- and 3-fold over baseline at the highest doses of heparin and argatroban, respectively (Fig. 3). However, there was no significant change in activated partial thromboplastin time at any of the doses of RPR208566 tested; activated partial thromboplastin time was only 1.3-fold over baseline at the highest dose of RPR208566. Prothrombin time increased dose-dependently in response to argatroban and heparin administration, with increases of 2.3- and 2.6-fold over baseline, respectively, at the highest doses (Fig. 3). Similar to activated partial thromboplastin time, there was no significant change in prothrombin time at any dose of RPR208566 tested. Coagulation times were similar at 15 (data not shown) and 75 min (Fig. 3) after drug administration, indicating that stable levels of anticoagulation were achieved with all agents tested.

Argatroban had no effect on template bleeding time (Fig. 4), while only modest increases of 1.8- and 1.5-fold over baseline were produced by heparin and RPR208566, respectively, at the maximum doses tested.

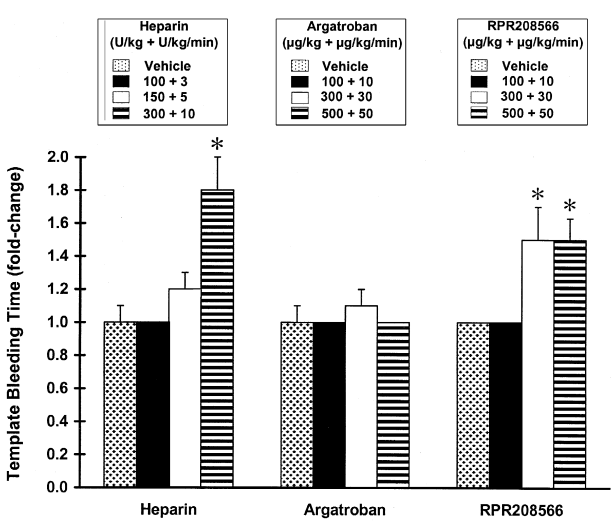

Fig. 4. Effect of RPR208566, argatroban, and heparin on template bleeding time during $FeCl_2$-induced thrombosis in the rat carotid artery. Data are shown as fold-changes from pre-drug control measurements. $n = 5$ per treatment group. $^*P < 0.05$ vs. vehicle.

4. Discussion

Thrombosis is the complex process involving changes in the vascular wall, platelet activation, adhesion, and aggregation, and initiation and propagation of the coagulation cascade. Thrombin plays a central role in this process and its regulation and activity have been studied in great detail in order to discover agents that would prevent thrombosis without substantially altering normal hemostasis. Heparin and heparin-like compounds have suffered from their requirement for co-factors and from their inability to inhibit clot-bound thrombin and factor Xa (Rosenberg and Damus, 1973; Hirsh and Levine, 1992). Although able to inhibit ''clot-bound'' thrombin, direct thrombin inhibitors may be limited in efficacy and safety because they inhibit only the activity of thrombin, allowing the production of thrombin to continue.

An alternative strategy is to prevent thrombin formation by inhibiting factor Xa, which plays a pivotal role in the coagulation cascade. Direct factor Xa inhibition has several potential advantages over direct thrombin inhibition. First, inhibition of factor Xa removes the source of thrombin and reduces the possibility of continued thrombus formation with termination of therapy. Factor Xa inhibition also permits the natural anticoagulant pathways, such as the thrombomodulin-activated Protein C pathway, to remain intact, thereby acting as a negative feedback mechanism to prevent further thrombin production. Finally, factor Xa inhibitors affect coagulation specifically without impairing platelet function, potentially resulting in an improved efficacy-to-safety ratio (Hara et al., 1995; Sato et al., 1998).

RPR208566, a potent, reversible, and direct inhibitor of factor Xa, produced a dose-dependent decrease in thrombus mass and in time-to-occlusion, with the maximal antithrombotic effect achieved at 500 μg/kg + 50 μg/kg/min. Heparin also produced a dose-dependent antithrombotic effect, albeit at very high plasma heparin concentrations. These results are similar to previous reports indicating that supratherapeutic doses of heparin are required to achieve maximal inhibition of thrombus forma-

tion in rat models of thrombosis (Schumacher et al., 1993; Finkle et al., 1998; Kawasaki et al., 1998).

Inhibition of thrombus formation achieved at the highest dose of RPR208566 was associated with < 1.5-fold increases in activated partial thromboplastin time, prothrombin time, and template bleeding time. These results are consistent with the minimal changes in these parameters that have been reported with other direct Xa inhibitors in similar models of thrombosis (Hara et al., 1995; Herbert et al., 1996; Kawasaki et al., 1998; Sato et al., 1998). In contrast, the highest doses of heparin or argatroban were associated with approximately 6- and 3-fold increases in activated partial thromboplastin time, respectively, and a 2.5-fold increase in prothrombin time was elicited by both drugs. These results are consistent with the notion that direct factor Xa inhibition may be able to prevent thrombus formation without creating a systemic hypocoagulable state. Another potential safety advantage of RPR208566 in acute thrombotic indications that might require surgical intervention is the short half-life of RPR208566 ($t_{1/2\beta} = 15$ min in rats), which would allow for easy reversal of the anticoagulant effect.

The concentration of a direct factor Xa inhibitor required to prolong clotting time in plasma-based assays (e.g., activated partial thromboplastin time, prothrombin time, and activated clotting time) is usually much greater than the K_i against the purified enzyme in a chromogenic assay (Hara et al., 1995; Taniuchi et al., 1998). The plasma-based clotting assays include all of the components of the enzymatic pathway that lead to explosive production of factors Xa and IIa. For example, prothrombinase-bound factor Xa is catalytically more efficient than free factor Xa (Mann, 1987; Hemker and Beguin, 1991). Under these conditions, greater concentrations of factor Xa inhibitors are required to prevent in vitro clot formation than are needed to prevent the cleavage of a chromogenic substrate by a purified enzyme.

None of the agents tested in these experiments produced dramatic increases in template bleeding time. For example, prolongation of template bleeding time at the highest doses of heparin and RPR208566 was 1.8- and 1.5-fold, respectively, over baseline. Argatroban had no effect upon bleeding time even at the highest dose tested. These relatively modest changes in bleeding times are consistent with other rat studies demonstrating less than 2-fold increases in bleeding time with similar agents (Schumacher et al., 1996; Morishima et al., 1997).

In conclusion, RPR208566 is a novel, potent inhibitor of coagulation factor Xa that inhibited thrombus formation in a well-established rat model at doses that did not significantly alter systemic coagulation (as measured by activated partial thromboplastin time and prothrombin time) or increase template bleeding time. RPR208566 was as effective as supratherapeutic doses of heparin and was at least as effective as equal doses of argatroban. This study suggests that factor Xa inhibitors, such as RPR208566, may provide safe and effective therapy for thrombotic vascular diseases.

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