

RPR120844, a Novel, Specific Inhibitor of Coagulation Factor Xa Inhibits Venous Thrombosis in the Rabbit

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Summary

The in vivo antithrombotic activity of RPR120844, a novel synthetic coagulation factor Xa (fXa) inhibitor (Ki = 7 nM), was assessed by its ability to inhibit thrombus formation in a damaged segment of the rabbit jugular vein. Intravenous dose-response studies were performed and thrombus mass (TM), activated partial thromboplastin time (APTT), prothrombin time (PT), inhibition of *ex vivo* fXa activity and plasma drug levels (PDL) were determined. TM, measured at the end of a 50 min infusion, was significantly reduced ($p < 0.05$ vs saline-treated animals) by RPR120844 at 30 and 100 μg/kg/min. At doses of 10, 30 and 100 μg/kg/min, APTT was prolonged by 2.1, 4.2 and 6.1-fold, and PT was prolonged by 1.4, 2.2 and 3.5-fold, respectively. PDL were determined by measuring anti-fXa activity using an amidolytic assay. Peak PDL were 0.8 ± 0.3, 1.5 ± 0.9 and 2.4 ± 0.6 μM, respectively. The drug effect was reversible with APTT, PT and PDL returning toward pretreatment values 30 min after termination of treatment. The results suggest that RPR120844, or similar compounds, may provide an efficacious, yet easily reversible, means of inhibiting thrombus formation.

Introduction

Activation of the coagulation system results in conversion of factor X to factor Xa (fXa). FXa in the presence of cofactor Va, calcium and phospholipid membranes forms the prothrombinase complex which converts prothrombin (factor II) to thrombin (factor IIa). Thrombin then cleaves fibrinogen to fibrin, culminating in local thrombus formation. Thrombin further promotes thrombosis by activating factor XIII, which stabilizes the fibrin-rich thrombus, by stimulating platelet aggregation and by positive feedback to the coagulation cascade via activation of cofactors V and VIII.

Unfractionated heparin and low molecular weight heparins exert their antithrombotic effect by complexing with antithrombin III (ATIII), and primarily inhibiting thrombin and fXa. However, clot-bound thrombin, in contrast to fluid-phase thrombin, is resistant to inactivation by heparin (1), perhaps because the heparin binding site on thrombin is masked while the enzyme is bound to fibrin. Because fibrin-bound thrombin mediates thrombus-associated procoagulant activity, the inability of heparin to inhibit fibrin-bound thrombin may be at least partly responsible for the limitations of heparin therapy. ATIII-independent thrombin inhibitors such as hirudin and D-phenylalanyl-L-prolyl-arginyl chloromethyl ketone (PPACK) have been shown to effectively inhibit thrombus-associated procoagulant activity (1), thus providing a mechanistic advantage over heparin-like compounds.

Recent studies have shown that prothrombinase-associated fXa activity, like fibrin-bound thrombin, significantly contributes to the procoagulant activity of whole blood clots in vitro, and may in fact play a more important role than thrombin in propagating thrombus formation (2, 3). When bound to the prothrombinase complex, fXa is resistant to inhibition by the ATIII-dependent anticoagulants, but not to inhibition by tick anticoagulant peptide (TAP) (3), suggesting that direct inhibition of fXa may effectively inhibit clot formation. Indeed, it has been shown that sustained inhibition of blood clot-associated procoagulant activity can be achieved by specific inhibition of fXa, but not by inhibition of thrombin (4). In vivo, selective inhibition of fXa by rTAP or antistasin (ATS) has been shown to effectively inhibit thrombus formation in several different animal models of thrombosis (5, 6, 7). More recently it was demonstrated that selective fXa inhibition by a single subcutaneous injection of recombinant nematode anticoagulant peptide (rNAP), significantly prolonged the time to coronary artery occlusion, reduced thrombus mass and reduced the incidence of death due to myocardial infarction in a canine model of coronary thrombosis (8).

Intensive research focused in the area of fXa inhibition has led to the discovery of several small synthetic inhibitors of fXa. DX9065a, an amidinonaphthyl derivative, has been reported to be a specific inhibitor of fXa with a Ki of 41 nM (9) and to inhibit thrombus formation in several animal species (10). A sulphated pentasaccharide, SR90107A/Org 31540, analogus to the pentasaccharide sequence in heparin with high affinity to antithrombin III has been reported to be a highly specific inhibitor of fXa and to inhibit venous thrombus formation in rabbits (11). Recently, YM-60828, a synthetic fXa inhibitor with a Ki of 1.3 nM, has been reported to inhibit venous thrombosis in a rat model, with little effect on bleeding time (12).

This report describes the in vivo antithrombotic efficacy of a novel synthetic coagulation fXa inhibitor, RPR120844, in a well established rabbit model of venous thrombosis.

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Material and Methods

RPR120844, [(7'-Methoxy-2-naphthalenesulfonyl)-methyl-amino]-2-oxo-pyrrolidin-3-yl-4-[2-(aminoiminomethyl)-methylthiophene]-amide hydrochloride (Fig. 1), was synthesized by the Medicinal Chemistry Department at Rhône-Poulenc-Rorer Pharmaceuticals, Collegeville, PA, USA.

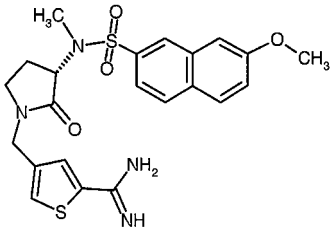

Fig. 1 Structure of RPR120844: [(7'-Methoxy-2-naphthalenesulfonyl)-methyl-amino]-2-oxo-pyrrolidin-3-yl-4-[2-(aminoiminomethyl)-methylthiophene]-amide hydrochloride

Enzyme Assays Using Chromogenic Substrates

Human factor Xa and thrombin were obtained from Enzyme Research Laboratories, Inc. (South Bend, Indiana). Bovine trypsin was obtained from Sigma Chemical Co. (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 fXa (American Diagnostica Inc., Greenwich, CT), Pefachrome TH, Pefachrome-tPA (Centerchem, Inc., Stamford, CT) and S-2765 and S-2366 (Diapharma Group Inc., Franklin, OH) for fXa, thrombin, tPA, trypsin, and plasmin and aPC, respectively.

All enzyme assays were carried out 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 preincubated 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). Under the experimental conditions, less than 10% of the substrate was consumed in all assays.

Animal Studies

All procedures in these studies were performed in accordance with the Animal Welfare Act Regulations and the Guide for the Care and Use of Laboratory Animals (13), and were approved by the Rhône-Poulenc Rorer Animal Care and Use Committee.

Male and female New Zealand White rabbits weighing between 2.9 and 3.5 kg were used. The rabbits were anesthestized with a mixture of Ketamine and Xylazine (50/10 mg/kg, i.m.) and supplemental anesthesia was administered by i.m. injection of the anesthetic mixture, as required. The right common jugular vein was cannulated with PE-90 for administration of vehicle or RPR120844. The right common carotid artery was cannulated with PE-90 for collection of blood samples. The left common jugular vein was identified and the bifurcation of the internal and external jugular veins was located and dissected free from the surrounding tissue. A 2 cm segment caudal to the bifurcation was isolated and any side branches draining into this segment were ligated. Sutures were put into place so that at a later time this segment could be ligated and removed.

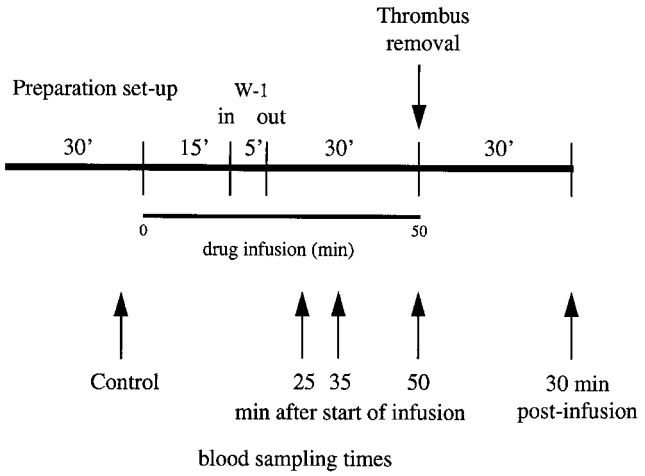

Fig. 2 Experimental protocol: The surgical preparation of the rabbit jugular vein thrombosis model is described in the Materials and Methods section. W-1: polyoxyethylene ether

The experimental protocol is presented in Fig. 2. A control blood sample was obtained and drug infusion (10, 30 or 100 μg/kg/min, 0.1 ml/min) or vehicle (saline, 0.1 ml/min) was started and continued for 50 min. Ten minutes after the start of drug or vehicle treatment, PE-90 was inserted into the internal jugular vein and passed into the common jugular vein for a distance of approximately 0.5 cm. The jugular vein segment was temporarily isolated from the rest of the circulation by occluding the external jugular vein just cephalad to the bifurcation of the internal and external jugular vein, and the common jugular vein approximately 2 cm caudal to the bifurcation, with non-traumatic vascular clamps. This segment was flushed gently with saline and then was filled with 0.5 ml of a 5 mg/ml solution of polyoxyethylene ether (W-1, Sigma) for 5 min. Polyoxyethylene ether is a detergent which disrupts the endothelial lining of the vessel thus creating a thrombogenic surface. A stenosis was created caudal to the jugular sac by tying a suture around the vein and an 18G needle. The needle was then removed so that blood flow could be restored through the narrowed vessel. The detergent was removed from the jugular segment and the segment was once again flushed with saline. The vascular clamps were then removed and the jugular segment was reexposed to flowing blood. Blood samples for determination of the activated partial thromboplastin time (APTT), prothrombin time (PT), anti-fXa activity and plasma drug levels were obtained 5 min prior to and then 25, 35 and 50 min after the start of drug treatment. A final blood sample was collected 30 min after the termination of drug treatment. At the termination of drug administration the jugular vein segment was ligated and removed. The thrombus was removed from the segment and its wet weight was recorded.

Blood samples (3 ml) were collected on cold, 3.8% trisodium citrate (1 vol citrate : 9 vols blood) and kept on ice. Plasma was separated from blood samples by centrifugation at 1500 × g (4° C) for 15 min. APTT was determined using an automated plasma coagulometer (MCA-210, Bio Data, Horsham, PA) and PT was determined with an MCA-210 automated plasma coagulometer. Aliquots of plasma were stored frozen at –70° C until assayed for fXa activity and drug plasma levels. Factor Xa activity was determined using a chromogenic assay (American Diagnostica) utilizing bovine fXa and Spectrozyme Xa. Optical densities were determined at a wavelength of 405 nm at 37° C using a Spectra Max 250, 96-well microplate spectrophotometer (Molecular Devices, CA). Plasma samples were diluted 1:2 with saline prior to addition to the well, and once added to the well, the plasma was diluted 1:10 (20 μl in a final volume of 200 μl) with the assay reagents. Inhibition of fXa activity was determined as follows: % inhibition of fXa activity = 1-(O.D. inhibitor/O.D. of the pre-drug control) × 100. Plasma drug concentrations were estimated from a standard curve which was prepared by adding varying concentrations of RPR120844 to control rabbit plasma and then plotting the concentration of RPR120844B against the % inhibition of fXa activity.

Statistics

The data are presented as the mean ± SEM. Statistical significance between treatment groups was tested using two-way analysis of variance. The least significant difference test (LSD) was used to perform multiple comparisons of means. Differences were considered significant when the p value was <0.05.

Results

In Vitro Studies

Chromogenic substrates were used to assess the inhibitory effect of RPR120844 on coagulation enzymes fXa and thrombin as well as other trypsin-like serine proteases. RPR120844 was a potent inhibitor

Table 1 Inhibitory activity (Ki) and selectivity of RPR120844 for fXa and selected serine proteases

Enzymes	Ki (nM)	Selectivity Ki_{enzyme}/Ki_{FXa}
fXa	7	1
Thrombin	1077	156
Trypsin	531	77
aPC	2391	342
Plasmin	4414	631
t-PA	>8681	>1240

Data are from single dose-response curves with each concentration tested in duplicate.

of fXa with a Ki = 7 nM. The Ki values for the other serine proteases were much higher, ranging from 530 nM to greater than 8600 nM. Table 1 shows that RPR 120844 is a potent and selective fXa inhibitor. In rabbit plasma, in vitro, the IC_{50} for the inhibition of fXa by RPR120844 was 27 nM.

In Vivo Studies

Thrombus mass was determined at the termination of the drug infusion. In rabbits treated with saline (n = 18), the mean thrombus mass was 42 ± 5 mg. Treatment with RPR120844 resulted in significant dose-dependent inhibition of thrombus mass (Fig. 3). At a dose of 10 μg/kg/min (n = 8), RPR120844 inhibited thrombus formation by 45% (23 ± 10 mg), and at 30 (n = 8) and 100 μg/kg/min (n = 8), thrombus mass was significantly reduced by 60% (17 ± 6 mg, $p < 0.05$) and 72% (12 ± 7 mg, $p < 0.05$, Fig 3), respectively.

The effect of RPR120844 on the activated partial thromboplastin time (APTT) and prothrombin time (PT) is presented in Fig. 4 A, B. In saline-treated rabbits, the APTT and PT were unaffected. APTT and PT were each dose-dependently prolonged by administration of RPR120844. When measured at the end of the drug infusion, RPR120844 (10 μg/kg/min) significantly prolonged APTT by 2.1 ± 0.3 times pre-drug control. Thirty minutes after termination of drug infusion, APTT had returned to values not significantly different from control (Fig. 4A). At 30 μg/kg/min and 100 μg/kg/min, RPR120844 produced significant extension of APTT (4.2- and 6.1-fold, respectively, when measured at the end of drug treatment, Fig. 4A). Thirty min following termination of the 30 μg/kg/min dose, the APTT was not significantly different from control values, indicating reversibility of the drug effect. At 100 μg/kg/min, the APTT returned from an approximate 6-fold increase at the end of the drug infusion to approximately a 2-fold increase 30 min following termination of treatment.

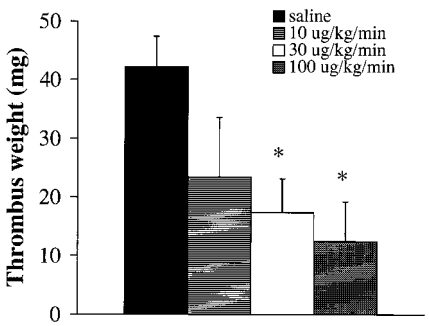

Fig. 3 Effect of vehicle (0.9% saline) and RPR120844 on thrombus weight in the anesthetized rabbit. Thrombus weight is expressed as mg wet weight. Values are the mean ± SEM. N = 18 for the saline-treated group, and 8 per dose for the RPR120844-treatment groups. * $p < 0.05$ vs. vehicle treated animals

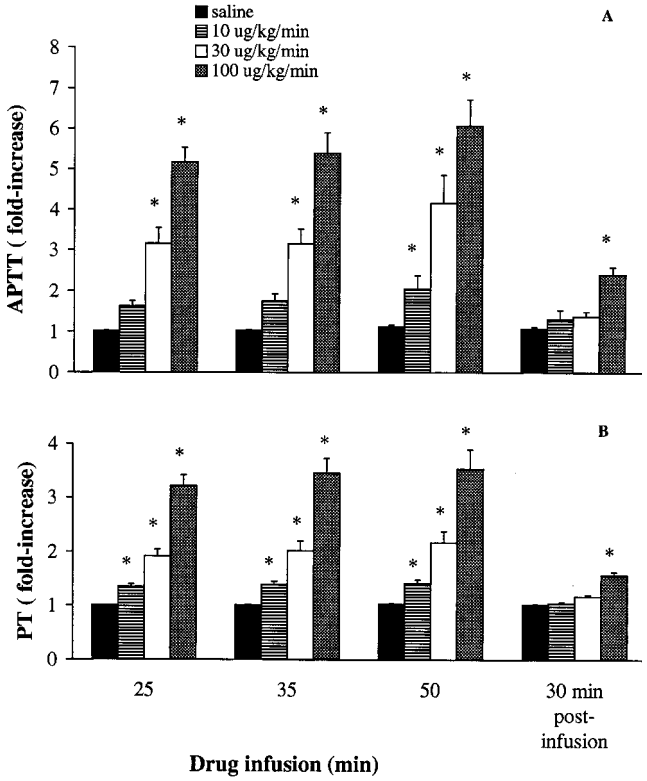

Fig. 4 Dose-response effects of RPR120844 on the activated partial thromboplastin time (APTT, panel A) and the prothrombin time (PT, panel B) in anesthetized rabbits. Drug was infused for 50 min. Blood samples were obtained at the indicated time points and the APTT and PT were determined using an automated plasma coagulometer as described in the methods section. Data are expressed as fold increases as compared to the pretreatment control values. The APTT and PT measured in plasma obtained from vehicle-treated animals remained unchanged at each time point. Pretreatment APTT and PT values ranged from a mean of 13.2 to 14.5 sec, and 9.6 to 10.4 sec respectively, and did not significantly differ among the treatment groups. Values are the mean ± SEM. N = 17 for the saline-treated group, and 8 per dose for the RPR120844-treatment groups. * $p < 0.05$ vs. pretreatment values

The PT response was qualitatively similar to the APTT response, but was less sensitive to treatment with RPR120844. For example, the peak change in PT observed with the highest dose administered was approximately 3.5-fold over baseline (Fig. 4B).

Infusion of RPR120844 produced a dose-dependent, reversible increase in the plasma concentration of the drug. Administration of RPR120844 at 10 μg/kg/min resulted in an estimated peak plasma level of 820 ± 250 nM. Thirty minutes after termination of the drug infusion, the plasma drug level had returned to near baseline (70 ± 30 nM, Fig. 5). At a dose of 100 μg/kg/min, RPR120844 administration resulted in a peak plasma level of 2440 ± 625 nM, which returned to 470 ± 20 nM 30 min after stopping drug treatment (Fig. 5).

Discussion

The results from this study show that RPR120844 is a potent (Ki fXa = 7 nM) and specific (Ki fIIa = 156 nM) inhibitor of coagulation fXa. Using a model of localized endothelial damage and stenosis in the jugular vein of rabbits, we have shown that specific inhibition of fXa significantly and dose-dependently inhibited venous thrombus formation and was associated with significant but transient increases in systemic

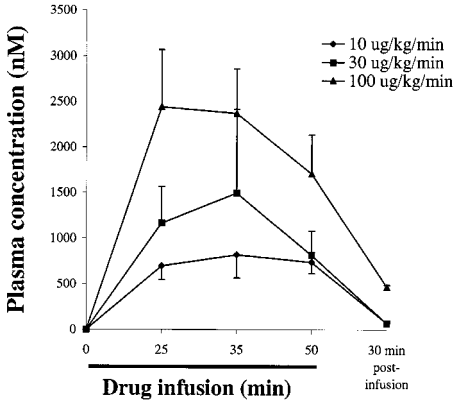

Fig. 5 Plasma drug concentration. RPR120844 plasma concentrations were estimated from a standard curve which was prepared by adding varying concentrations of RPR120844 to control rabbit plasma and then plotting the concentration of RPR120844B against the % inhibition of fXa activity as described in the methods section. These concentrations were then multiplied by the dilution factor to estimate the actual drug concentration in the plasma. Factor Xa activity was determined using a chromogenic assay (American Diagnostica) utilizing bovine factor Xa and Spectrazyme Xa. Optical densities were determined at a wavelength of 405 nm at 37° C. The inhibition of factor Xa activity was determined as follows: % inhibition of factor Xa activity = 1-(O.D. inhibitor/O.D. of the pre-drug control) × 100. Plasma drug levels in saline treated animals were zero. Values are the mean ± SEM. N = 2-5 per treatment group

anticoagulation, as measured by APTT and PT. Plasma drug levels, estimated from an *ex vivo* bioassay, demonstrated dose-dependent increases that were readily reversible following termination of drug administration.

While RPR120844 possesses an apparently short half-life, recent studies suggest that fXa inhibition may provide prolonged antithrombotic activity even after termination of drug administration. For example, sustained inhibition of whole-blood clot procoagulant activity by inhibiting thrombus associated fXa with rTAP, *in vitro*, has been reported (4). Kotzé et al. extended this observation to an *in vivo* setting, demonstrating long-term antithrombotic effects in baboons by transient inhibition of fXa with rTAP (14). These results suggest that it might be possible to inhibit thrombus formation with only transient inhibition of fXa activity, which may be beneficial in a clinical setting where it may be necessary to rapidly reverse anticoagulant activity prior to emergency surgical interventions. Whether these observations can be extended to small synthetic inhibitors of fXa, such as RPR120844, remains to be determined.

Of possible concern in this study is the effect of RPR120844 on APTT. At a dose of 100 μg/kg/min, RPR120844 caused a 6-fold increase in APTT. While this may represent a high degree of systemic anticoagulation, the effect was transient, and significant inhibition of thrombus formation was observed at 30 μg/kg/min, a dose which caused only 4-fold increase in APTT. Interestingly, when specific fXa inhibitors are examined at effective antithrombotic doses, some investigators report marked increases in APTT (5, 8, 15), but some do not (6, 7, 9). It is possible that these differential effects may be attributed to species differences, different drug doses or different assay conditions. It is also conceivable that fXa inhibitors have inherent, and perhaps subtle, variations in the way they interact with fXa that produce distinct effects on coagulation assays. The specific mechanisms responsible for any such differences have yet to be determined, but may be important for the discovery of novel anticoagulant compounds which possess a high benefit-to-risk ratio.

In summary, we have shown that infusion of RPR120844, a novel, specific coagulation fXa inhibitor, dose-dependently inhibited thrombus formation and reversibly inhibited markers of systemic coagulation in a rabbit model of acute venous thrombosis. Substantial antithrombotic effects were observed when the APTT and PT were prolonged only 2-fold or less. The results suggest that RPR120844 or similar compounds may provide safe and effective therapy for thrombotic indications.

References

1. Weitz JI, Hudoba M, Massel D, Maraganore J, Hirsh J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by anti-thrombin III-independent inhibitors. J Clin Invest 1990; 86 (August): 385-91.
2. Prager NA, Abendschein DR, McKenzie CR, Eisenberg PR. Role of thrombin compared with factor Xa in the procoagulant activity of whole blood clots. Circulation 1995; 92: 962-7.
3. Eisenberg PR, Siegel JE, Abdenschein DR, Miletich JP. Importance of factor Xa in determining the procoagulant activity of whole-blood clots. J Clin Invest 1993; 91 (May): 1877-83.
4. McKenzie CR, Abendschein DR, Eisenberg PR. Sustained inhibition of whole-blood clot procoagulant activity by inhibition of thrombus-associated factor Xa. Arterioscler Thromb Vasc Biol 1996; 16: 1285-91.
5. Schaffer LW, Davidson JT, Vlasuk GP, Dunwiddie CT, Sigel PKS. Selective factor Xa inhibition by recombinant antistasin prevents vascular graft thrombosis in baboons. Arteriosclerosis and Thrombosis 1992; 12: 879-85.
6. Nicolini FA, Lee P, Malycky JL, Lefkovits J, Kottke-Marchant K, Plow EF. Selective inhbition of factor Xa during thrombolytic therapy markedly improves coronary artery patency in a canine model of coronary thrombosis. Blood Coagul Fibrin 1996; 7: 39-48.
7. Vlasuk GP, Ramjit D, Fujita T, Dunwiddie CT, Nutt EM, Smith DE. Comparison of the in vivo anticoagulant properties of standard heparin and the highly selective factor Xa inhibitors antistasin and Tick Anticoagulant Peptide (TAP) in a rabbit model of venous thrombosis. Thromb Haemost 1991; 65: 257-62.
8. Rebello SS, Blank HS, Rote WE, Vlasuk GP, Lucchesi BR. Antithrombotic efficacy of a recombinant nematode anticoagulant peptide (rNAP5) in canine models of thrombosis after single subcutaneous administration. J Pharm Exp Ther 1997; 283: 91-9.
9. Hara T, Yokoyama A, Ishihara H, Yokoyama Y, Nagahara T, Iwamoto M. DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa. Thromb Haemost 1994; 71: 314-9.
10. Herbert JM, Bernat A, Dol R, Herault JP, Crepon B, Lormeau JC. DX 9065, a novel, synthetic, selective and orally active inhibitor of factor Xa: In vitro and in vivo studies. J Pharm Exp Ther 1996; 276: 1030-8.
11. Herbert JM, Petitou M, Lormeau JC, Cariou R, Necciari J, Magnani HN. SR 90107A/Org 31540, a novel anti-factor Xa antithrombotic agent. Cardiovascular Drug Reviews 1997; 15 (1): 1-26.
12. Sato K, Kawasaki T, Hisamichi N, Taniuchi Y, Hirayama F, Koshio H. Antithrombotic effects of YM-60828, a newly synthesized factor Xa inhibitor, in rat thrombosis models and its effects on bleeding time. Brit J Pharmacol 1998; 123: 92-6.
13. National Research Council. Guide for the care and use of laboratory animals. Washington, D.C.: National Academy Press, 1996.
14. Kotze HF, Lamprecht S, Badenhorst PN, Roodt JP, vanWyk V. Transient interruption of arterial thrombosis by inhibition of factor Xa results in long-term antithrombotic effects. Thromb Haemost 1997; 77: 1137-42.
15. Lyle EM, Fujita T, Conner MW, Connolly TM, Vlasuk GP, Lynch JL, Jr. Effect of inhibitors of factor Xa or platelet adhesion, heparin, and aspirin on platelet deposition in an atherosclerotic rabbit model of angioplasty injury. J Pharmacol Toxicol Meth 1995, 33: 53-61.

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