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Antithrombotic and hemostatic capacity of factor Xa versus thrombin inhibitors in models of venous and arteriovenous thrombosis

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Abstract

Thrombin plays a central role in venous and arterial thrombosis. We utilized two different rabbit models of in vivo thrombosis to investigate the effect of inhibitors of thrombin generation and thrombin activity. The agents tested were specific inhibitors of factor Xa (fXa) [*N*2-[(phenylmethyl)sulfonyl]-D-arginyl-*N*-[(1*S*)-4-[(aminoiminomethyl)amino]-1-(2-thiazolylcarbonyl)butyl]-glycinamide (C921-78)] and thrombin [D-phenylalanyl-*N*-[4-[(aminoiminomethyl)amino]-1-(chloroacetyl)butyl]-L-prolinamide (PPACK)], as well as drugs that affect both thrombin and fXa, unfractionated and low molecular weight (enoxaparin) heparin. The agents administered as constant intravenous infusion were evaluated for antithrombotic efficacy in anesthetized rabbits. All four agents were capable of dose dependent inhibition of thrombosis in venous and arteriovenous thrombosis models. However, due to the more aggressive nature of thrombotic stimulation in the arteriovenous shunt model, complete cessation of thrombus growth was not achieved for any of the agents at the doses tested. Comparison between the agents focused on the differences in extension of coagulation parameters (activated partial thromboplastin time, prothrombin time, thrombin clotting time), changes in hematological parameters, and extension of rabbit cuticle bleeding time at doses required to produce maximum inhibition in the thrombosis models. In the venous thrombosis model at the maximally effective dose, C921-78 had minimal extension of ex vivo clotting parameters, while enoxaparin and unfractionated heparin demonstrated a two to sevenfold increase in activated partial thromboplastin times, and PPACK had a threefold extension of thrombin clotting times. In addition, unlike the other three agents, which exhibited no significant changes in hematological parameters, PPACK demonstrated dose dependent thrombocytopenia. A standardized cuticle bleeding time was used as a measure of perturbation of hemostasis. The agents were evaluated for significant increases in bleeding time at doses up to eight times that needed to completely inhibit venous thrombus formation. Unfractionated heparin displayed a significant bleeding time effect at the dose required to inhibit venous thrombosis (100 u/kg + 2 u/kg/min). Enoxaparin and PPACK caused significant bleeding time extensions at four times the fully efficacious venous dose (800 u/kg + 8 u/kg/min and 30 μg/kg/min). By contrast, C921-78 did not significantly increase bleeding time even at eight times the maximally effective dose (240 μg/kg + 7.2 μg/kg/min). Our results demonstrate that specific inhibition of fXa can be utilized to derive potent antithrombotic activity without disrupting extravascular hemostasis.

Keywords: Thrombosis; Factor Xa; Thrombin; Coagulation inhibitor

1. Introduction

Thrombin, a plasma protease, plays a key role in both platelet activation and thrombus formation. It has been postulated that efficient regulation of thrombosis may be achieved by regulation of thrombin. The current strategies employed involve reduction of thrombin generation as well as inhibition of the proteolytic activity of thrombin. The prothrombinase complex is the sole site of thrombin formation in the vasculature (Mann et al., 1990). The complex is comprised of factor Xa (fXa) assembled with cofactor Va in the presence of Ca^{2+} on the surface of activated platelets. At present, there are no prothrombinase/fXa inhibitors approved for clinical use. Synthetic inhibitors of fXa and thrombin have been the targets of drug development. In animal models of thrombosis, antithrombotic efficacy has been achieved by inhibition of catalytic activity of fXa [tick anticoagulant peptide (Lynch et al., 1995), *N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]

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phenyl]*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl] acetic acid dihydrochloride (YM-60828) (Kawasaki et al., 1998), (+)-(2*S*)-2-[4-[[(3*S*)-1-acetimidoyl-3-pyrrolidinyl]oxy]-phenyl]-3-[7-amidino-2-naphthyl] propanoic acid hydrochloride pentahydrate (DX-9065a) (Hara et al., 1995), or by disruption of prothrombinase complex assembly [catalytically inactive fXa (Hollenbach et al., 1994; Wong et al., 1997)]. Inhibitors of thrombin activity (Hirudin, Hirulog, PPACK, boroarginine derivatives) have also fared well by similar criteria. Comparison of data from different animal models is made difficult by the fact that the event triggering thrombosis and location of thrombi vary widely in the different protocols. Furthermore, the evaluation criteria for measurement of antithrombotic and antihemostatic effects is not comparable between studies. In this study, we compared the antithrombotic effects of a direct anti thrombin [D-phenylalanyl-*N*-[4-[(aminoiminomethyl)-amino]-1-(chloroacetyl)butyl]-L-prolinamide (PPACK)], a direct anti fXa [*N*2-[(phenylmethyl)sulfonyl]-D-arginyl-*N*-[(1*S*)-4-[(aminoiminomethyl)amino]-1-(2-thiazolylcarbonyl)butyl]-glycinamide (C921-78)] and two antithrombin III mediated inhibitors, unfractionated heparin (which contains equivalent amounts of anti fXa and antithrombin inhibitory polysaccharides) and enoxaparin (which contains anti fXa and antithrombin polysaccharides in a 3:1 ratio). The comparison was carried out in two separate in vivo models in anesthetized rabbits. These models evaluate antithrombotic efficacy by monitoring thrombus accretion under venous and arteriovenous blood flow conditions. Antihemostatic parameters were evaluated by classical clotting assays such as activated partial thromboplastin times as well as in a standardized rabbit cuticle bleeding time model.

2. Materials and methods

Enoxaparin (Lovenox, manufactured by Rhone Poulenc Rorer) and unfractionated heparin (Heparin sodium injection, Elkins-Sinn) were purchased from a medical supplies company. PPACK was purchased from Calbiochem Activated partial thromboplastin time was determined using actin FS (Activated PTT reagent, Dade) and calcium on an automated coagulation timer (ACL 3000, Automated Coagulation Laboratory). Prothrombin times and fibrinogen were evaluated using Thromboplastin C Plus (Dade). Thrombin clotting reagent (Dade) was used for determination of thrombin clotting times. Plasma concentrations of low molecular weight heparin and unfractionated heparin were quantitated by Coatest LMW heparin/heparin (Chromogenix).

2.1. Details of in vitro inhibitory assays

All assays measured proteolytic activity of enzyme by following cleavage of paranitroanilide (pNA) substrate in a 96-well microtiter plate. The proteases (supplier) and corresponding substrates are as follows: human activated Protein C (Haematologic Technologies); *H*-D-Lys(γCbo)-Pro-Arg-pNA, human pancreatic chymotrypsin (Athens Research Technologies); *N*-Suc-Ala-Ala-Pro-Phe-pNA, porcine pancreatic elastase (Sigma); *N*-Suc-Ala-Ala-Ala-pNA, human Factor IXa (Enzyme Research Laboratories); *Z*-D-Arg-Gly-Arg-pNA, human fXa (Haematologic Technologies); *Z*-D-Arg-Gly-Arg-pNA, human Factor XIa (Haematologic Technologies); Pyr Glu-Pro-Arg-pNA, human Factor XIIa (Enzyme Research Laboratories); *Z*-D-Arg-Gly-Arg-pNA, human plasma kallikrein (Enzyme Research Laboratories); Benz-Pro-Phe-Arg-pNA, human plasmin (Haematologic Technologies); Tosyl-Gly-Pro-Lys-pNA, recombinant human tissue plasminogen activator (Genentech); N-$MeSO_2$-D-Phe-Gly-Arg-pNA, human α-thrombin (Haematologic Technologies); Tosyl-Gly-Pro-Arg-pNA. A series of inhibitor samples (0–500 μM) were added to wells in a microtiter plate containing tris buffered saline pH 7.5/0.1% bovine serum albumin/5 mM calcium chloride. After addition of enzyme, the plate was incubated for 5 min at room temperature. Then the appropriate amidolytic substrate was added at a concentration of 100 μM/well to result in a final volume of 200 μl/well. The rate of change in absorbance at 405 nm was monitored by a Softmax plate reader (Molecular Devices). Numerical analysis for determination of IC_{50} (inhibitor concentration required for half maximal inhibition) was carried out by fitting experimental data on a four-parameter curve.

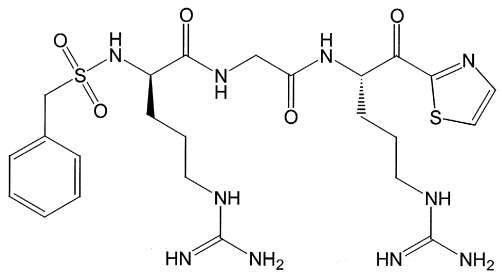
2.2. Plasma bioassay for C921-78

Bioactivity of inhibitor was determined after separation of free inhibitor from the fraction bound to plasma proteins. Citrated plasma samples (stored at −80°C) from rabbits were thawed prior to assay. Samples were diluted in buffered physiologic saline and ultrafiltered using Microcon 10 by centrifugation at 4°C (30 min at 16,000 × *g*). Filtrates were assayed for human fXa inhibitory activity in a 96 well format. Proteolytic activity of fXa was quantitated by measuring the extent of cleavage of S-2765 (*Z*-D-Arg-Gly-Arg-pNA, Diapharma). The standard curve of inhibitor concentration was generated by addition of known amounts of inhibitor into pooled rabbit plasma. The standards were processed by the same filtration protocol as the actual experimental samples. The lowest level of quantitation for C921-78 was at a plasma concentration of 2 ng/ml.

2.3. Effect of intravenous administration in rabbits

The deep vein thrombosis model and the arteriovenous shunt model require steady state infusion of drug during thrombus formation. The infusion rate for the two models was based on the following pharmacokinetic studies. Male New Zealand white rabbits, 3.2–3.8 kg, were anesthetized

Table 1



C921-78

Enzyme	IC_{50} (μM)
Factor Xa	0.0005
Thrombin	7.4
Activated Protein C	7.5
Factor IXa	0.659
Factor XIa	0.250
Factor XIIa	0.050
Plasma Kallikrein	0.014
Tissue plasminogen activator	0.214
Plasmin	0.526
Chymotrypsin	> 500
Elastase	> 500

by intramuscular administration of ketamine cocktail [a mixture of 75 mg/kg ketamine hydrochloride (Fort Dodge Laboratories), 8 mg/kg Xylazine (Vetus Animal Health) and 1.5 mg/kg Acepromazine (Fort Dodge Laboratories)]. A femoral vein was cannulated for blood sampling and a marginal ear vein was catheterized for compound administration. For determination of half-life of plasma clearance, an intravenous bolus dosing followed by serial blood sampling was employed. The elimination rate constant (k) was calculated from a logarithmic plot of the plasma concentration versus the time course of sampling in which the elimination half life ($T_{1/2}$) was calculated from drug elimination rate constant k ($T_{1/2} = 0.693/k$). Basic hematological parameters (hematocrit, white blood cell, red blood cell, and platelet counts) of non-fractionated whole blood samples were analyzed on an automated hematology analyzer (Coulter Microdiff).

2.4. Rabbit deep vein thrombosis model

The experimental protocol has been described in detail (Hollenbach et al., 1994). Briefly, a non-occlusive fibrin-rich thrombus was formed on a thrombogenic surface (cotton threads attached to copper wire) introduced into the abdominal vena cava via the femoral vein of an anesthetized rabbit. For measurements of antithrombotic activity at a steady state of plasma concentration, a continuous infusion was preceded by bolus administration. Inhibition of thrombus formation was determined by weight of harvested thrombi. Citrated plasma samples were obtained at −30, 0, 15, 30, 60, 90, and 120 min. Blood samples obtained during drug administration were used for measurements of plasma concentration, coagulation, and hematological parameters.

2.5. Rabbit arteriovenous shunt model

The experimental protocol has been described in detail (Wong et al., 1997). In anesthetized rabbits, an indwelling catheter was placed in a marginal ear vein for injection of drug, and the right femoral vein was catheterized for blood sampling. The left femoral artery and vein were isolated, cannulated with polyethylene tubing and connected with a polyethylene shunt containing a 6-cm long cotton thread. Experimental agent was administered as a bolus followed by a constant infusion. Dosing was initiated 15 min prior to shunt placement and continued during the experimental protocol. The cotton thread was removed 15 min after blood began to circulate through the shunt, and the thrombus-coated thread was weighed. Before thread removal, patency of the shunt was monitored by opening the venous end of the shunt and checking for blood flow. Under these experimental conditions, none of the shunts occluded, allowing all thrombus-coated threads to be used in the comparison of control or drug-treated animals. Blood samples obtained during drug administration were used for determination of plasma concentration, coagulation, and hematological parameters.

2.6. Rabbit bleeding time measurements

Cuticle bleeding time measurements were performed during steady state administration of antithrombotic agent or physiologic saline in anesthetized rabbits. In brief, blood from cuticles transected 4–5 mm from its base was gently ''wicked'' onto blotting paper by capillary action every 30 s until cessation of bleeding was observed (Hollenbach et al., 1994). Two cuticle bleeding time measurements were carried out prior to drug administration and three determinations were done during the 120 min of steady state infusion. Average of the two pre-infusion bleeding times were compared to average of the three times during drug administration and fold increase in cuticle bleeding time was calculated per animal. Blood samples obtained during drug administration were used for

Table 2
Plasma concentration required for twofold extension of ex vivo clotting

	C921-78 (μM)	Enoxaparin (U/ml)	Unfractionated heparin (U/ml)	PPACK (μM)
Activated partial thromboplastin time	0.84	3.2	0.47	3.7
Prothrombin time	7	> 1000	10.2	19.9
Thrombin clotting time	31	1.7	0.4	0.23

determination of plasma concentration, coagulation, and hematological parameters.

3. Results

3.1. Inhibitory effect of C921-78 on select serine proteases

C921-78 is a highly potent inhibitor of human fXa (Scarborough, 1998; Betz et al, 1999). As shown in Table 1, half-maximal inhibition of 1 nM human fXa is achieved at a subnanomolar concentration of C921-78. The inhibitory activity was highly selective for fXa, closely related proteases such as thrombin and activated protein C are effectively inhibited at much higher concentrations. The anticoagulant effect of C921-78 in ex vivo clotting assays such as activated partial thromboplastin time and prothrombin time were also found to be dose dependent (Table 2).

3.2. Deep vein thrombosis model

Histological analysis of the thrombus obtained in this model show that each was characterized by an irregular mosaic pattern of loosely organized fibrin strands (40% of clot cross-section) occurring as islands within tightly ag-

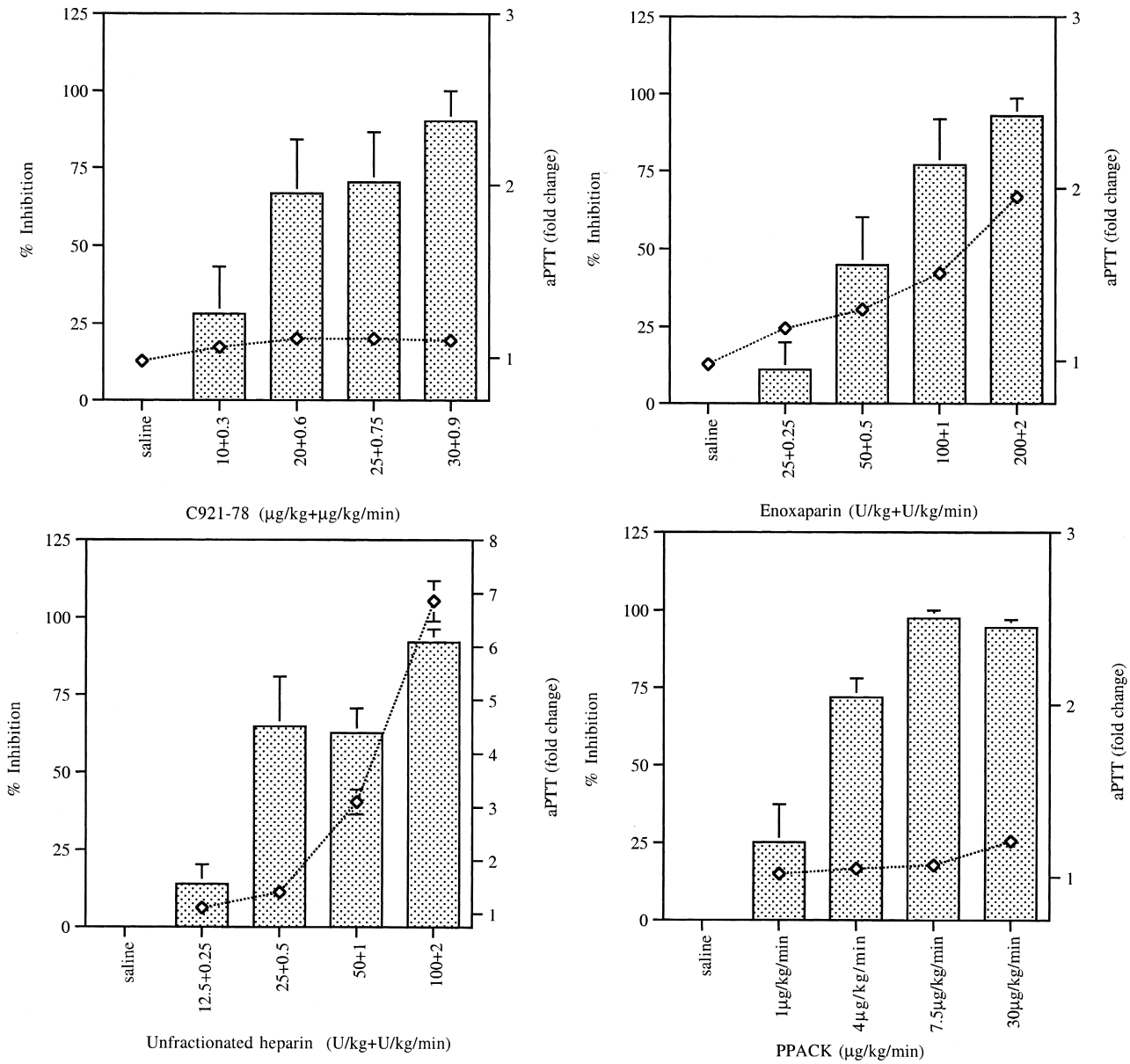


Fig. 1. Relative antithrombotic activities of C921-78, enoxaparin, heparin, and PPACK in a rabbit deep vein thrombosis model. The model measures inhibition of thrombus accretion on cotton threads placed in the abdominal vena cava of anesthetized rabbits. Details are described in Hollenbach et al. (1994). The bar graphs represent mean inhibition of thrombosis ± standard error of the mean. A minimum of six animals was studied for each dosage and for saline control. Activated partial thromboplastin times (aPTT) represented by (–◇–) are ex vivo measurements (mean of duplicate measurements ± standard error of the mean).

gregated and distorted erythrocytes (60% of clot cross-section). Leukocytes were uniformly distributed throughout, but platelet aggregates were not observed (Hollenbach et al., 1994). The present study compared the relative efficacy of C921-78, enoxaparin, unfractionated heparin, and PPACK. All agents inhibited thrombus formation in a dose-dependent manner (Fig. 1). Fig. 1 also depicts the average activated partial thromboplastin time for sampled rabbit plasma during the time course of drug infusion. Among the different coagulation parameters measured (activated partial thromboplastin time, prothrombin time, fibrinogen, and thrombin clotting time), extension of activated partial thromboplastin time displayed the best dose dependent correlation to plasma concentrations of C921-78 and the two heparins. At the highest dose of C921-78 (30 μg/kg + 0.9 μg/kg/min), activated partial thromboplastin time values had a 10% extension. By contrast, doses of enoxaparin and unfractionated heparin that completely inhibited thrombus growth had extended activated partial thromboplastin time by 1.95- and 6.7-folds, respectively. For evaluation of the effect of PPACK on rabbit ex vivo clotting times, thrombin clotting time had the greatest dose dependent increase. The dose of PPACK that demonstrates full antithrombotic activity extended thrombin clotting times by 2.1-fold over baseline values, the accompanying change in activated partial thromboplastin time was 7% (Hollenbach et al., 1994). In addition, PPACK produced a dose dependent thrombocytopenia with the highest dose (30 μg/kg/min) inducing an 82% reduction in platelet count (Hollenbach et al., 1994). Unfractionated heparin also caused dose dependent extensions in prothrombin time and thrombin clotting time (Hollenbach et al., 1994). The plasma levels of C921-78 were measured by determination of anti human fXa inhibitory activity and were linearly proportional to increasing doses (Table 3 and unpublished data).

3.3. Arteriovenous shunt model

We also compared the efficacy of all four agents in a model mimicking arterial thrombosis. Histopathological evaluation of thrombi produced in the arteriovenous model demonstrated similar irregular patterns of loosely organized fibrin lakes (30% of clot cross-section) to aggregated erythrocytes (70% of clot cross-section) as with the deep vein thrombosis model. However, the most notable difference observed with the arteriovenous model were numerous platelet aggregates seen throughout the fibrin lakes. The majority of platelet accumulations were composed of a significant proportion of partially degranulated platelets (Wong et al., 1997). All fXa and thrombin inhibitors examined in this model inhibited thrombus accretion in a dose-dependent manner (Fig. 2). Compared to inhibition of venous thrombosis, higher doses of agents were required to produce significant antithrombotic effects in the shunt model. However, even at the highest doses examined, none of the agents were capable of complete inhibition of thrombus accumulation. Due to the higher doses of drug utilized in this model, the increases in ex vivo coagulation parameter were exacerbated compared to the deep vein thrombosis model. Activated partial thromboplastin time changes at the highest dose used in this model for C921-78, enoxaparin, unfractionated heparin, and PPACK were 1.62-, 4.86-, 6.58-, and 1.29-folds, respectively.

3.4. Rabbit bleeding time model

The relationship between drug administration and extension of cuticle bleeding time is depicted in Fig. 3. The comparison was carried out at the dose that fully abolished thrombus accretion in the rabbit vena cava and at doses

Table 3
Plasma concentration and clotting times in rabbit bleeding time studies

Agent	*n*	Dose (bolus + infusion)	Activated partial thromboplastin time fold change (Mean ± S.E.M)	Plasma level (Mean ± S.E.M)
Saline	6	55 μl/min	0.98 ± 0.01	–
C921-78	6	30 μg/kg + 0.9 μg/kg/min	1.08 ± 0.03	26.30 ± 3.48 ng/ml
	6	120 μg/kg + 3.6 μg/kg/min	1.54 ± 0.05	143.59 ± 11.41 ng/ml
	6	240 μg/kg + 7.2 μg/kg/min	1.58 ± 0.06	255.38 ± 11.91 ng/ml
Enoxaparin	6	200 U/kg + 2 U/kg/min	2.10 ± 0.07	4.01 ± 0.24 U/ml
	6	800 U/kg + 8 U/kg/min	> 6	11.7 + 0.54 U/ml
	6	1600 U/kg + 16 U/kg/min	> 6	19.78 + 0.83 U/ml
Unfractionated heparin	8	100 U/kg + 2 U/kg/min	> 6	2.57 + 0.10 U/ml
	6	400 U/kg + 8 U/kg/min	> 6	9.82 + 0.40 U/ml
	6	800 U/kg + 16 U/kg/min	> 6	14.99 + 0.60 U/ml
PPACK	7	7.5 μg/kg/min	1.17 + 0.54	ND[a]
	6	30 μg/kg/min	1.50 + 0.67	ND[a]

[a] ND = Not determined.

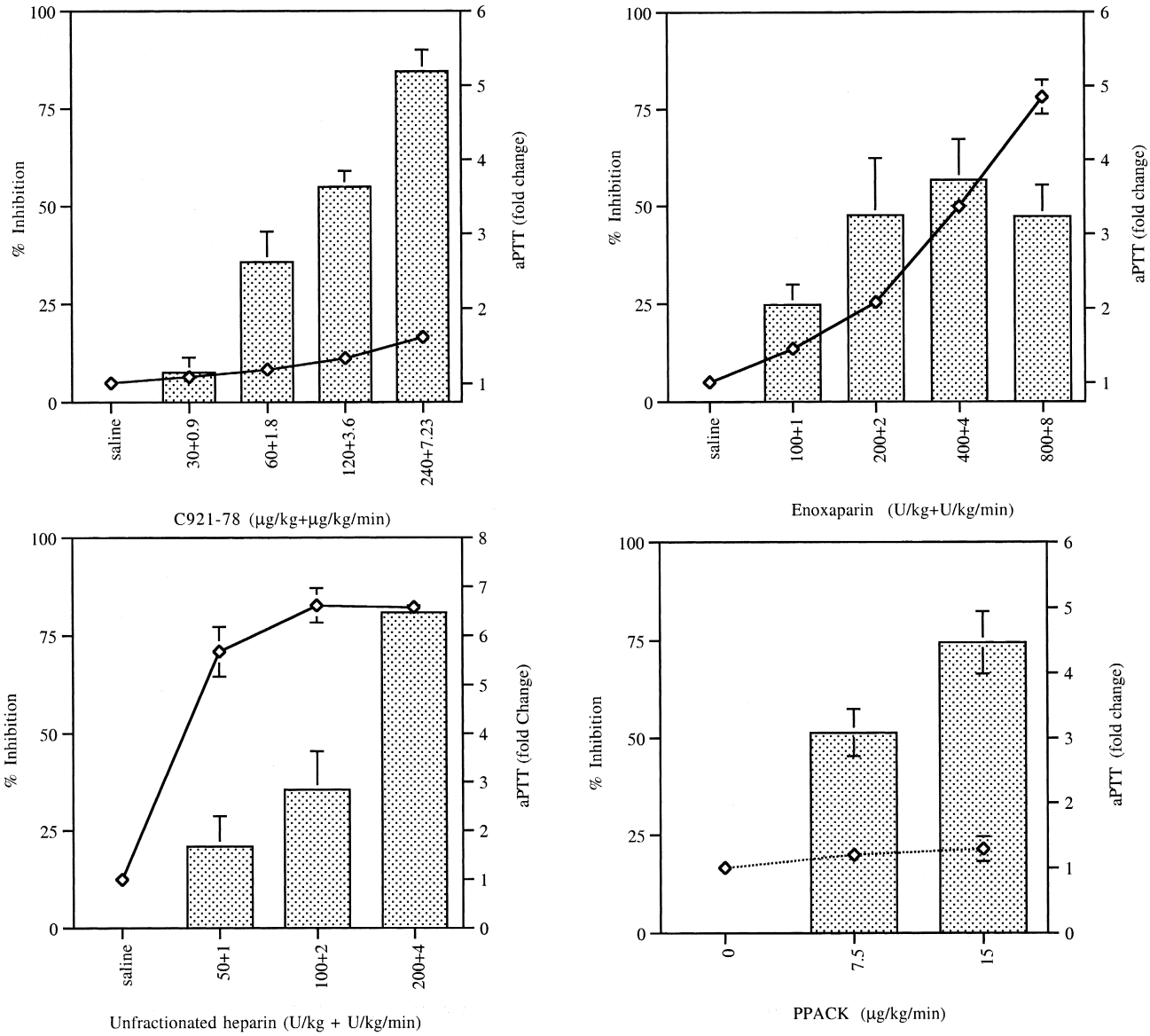


Fig. 2. Relative antithrombotic activities of C921-78, enoxaparin, heparin, and PPACK in a rabbit arteriovenous shunt model. The model measures inhibition of clot accumulation on cotton thread placed in a polyethylene shunt connecting the femoral artery and vein. Details are described in Wong et al. (1997). Drug treatment began 15 min prior to shunt placement. The bar graphs represent mean inhibition of thrombosis ± standard error of the mean. A minimum of six animals was studied for each dosage and for saline control. Activated partial thromboplastin times (aPTT) represented by (–◇–) are ex vivo measurements (mean of duplicate measurements ± standard error of the mean).

that were four and eightfold higher. We chose a maximum of 30 min for measurement of cuticle bleeding time. If the cuticle was still bleeding at 30 min, it was stopped with chemical cautery and the bleeding time recorded as 30 min. With the average baseline bleeding time of approximately 5 min, the maximum bleeding time due to administration of an inhibitor would therefore be limited to a sixfold increase. At the minimum dose that demonstrates $>90\%$ antithrombotic activity in the venous thrombosis model, C921-78, enoxaparin and PPACK did not significantly extend bleeding time above baseline measurements (Fig. 3). However cuticle bleeding time of unfractionated heparin treated animals at equivalent antithrombotic dose was significantly elevated. As seen in Fig. 3, even when the administered dose of C921-78 was eightfold higher, it did not result in a statistically significant extension of bleeding time. Unlike C921-78, enoxaparin at both four and eight times the dose had significant elevations in bleeding time. Due to an almost sixfold extension of bleeding time, dose escalation of PPACK was discontinued at the fourfold higher dosing regimen. Table 3 depicts the circulating level of inhibitors as quantitated in citrated plasma samples obtained during drug infusion. Table 3 also illustrates the extension of activated partial thrombo-

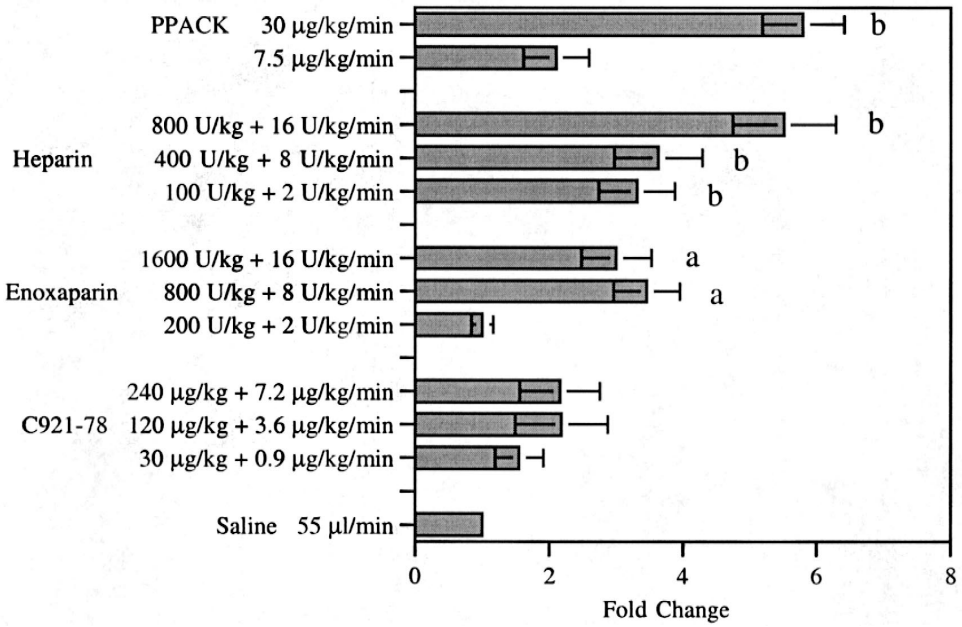


Fig. 3. Effect of antithrombotic regimen on rabbit cuticle bleeding times. Details are described in Hollenbach et al. (1994) and Wong et al. (1997). Drug doses correspond to 1, 4, and 8 times the fully effective antithrombotic dose in the rabbit deep vein thrombosis model (see Fig. 1). Baseline values are an average of two measurements taken prior to drug administration. All experimental bleeding times are an average of determinations at 5, 40 and 75 min during infusion of saline or experimental agent. Each bar represents fold change in bleeding time (mean ± standard deviation). $^{a}P < 0.05$ and $^{b}P < 0.01$ by Student's *t*-test as compared to baseline.

plastin time values at the higher doses of unfractionated heparin and enoxaparin.

4. Discussion

The cascade of events leading to platelet aggregation and blood coagulation is primarily responsible for thrombosis in arterial and venous settings. Thrombin's pivotal role in both platelet activation and blood clotting has made it a suitable target for antithrombotic agents. Inhibition of thrombin generation and thrombin activity has been studied in numerous animal models. However, it has been difficult to compare the relative antithrombotic efficacy of different agents because they have been studied under varying in vivo protocols. We were particularly interested in comparing agents that inhibit clotting factors by different mechanisms. C921-78 is a specific inhibitor of fXa. It inhibits human fXa with an inhibition constant (K_i) of 14 pM, the inhibitory constant against human thrombin is 1.6 μM (Betz et al., 1999). PPACK is a potent irreversible inhibitor of thrombin. Even at nanomolar concentrations, PPACK reacts stoichiometrically (1:1) to inactivate thrombin within a few minutes (Kettner and Shaw, 1979). It is also at least three orders of magnitude less reactive with other trypsin like proteases such as fXa (Kettner and Shaw, 1981). In addition to these two small molecule inhibitors, we also evaluated two different heparins whose inhibitory activity is mediated by the plasma serine protease inhibitor antithrombin III. Even though antithrombin III is present in blood in micromolar concentrations and is not a limiting factor, neither heparin can mediate inhibition of clot bound fXa or thrombin (Teitel and Rosenberg, 1983; Weitz et al., 1990). The thrombus also contributes to additional inhibitory pathways that compromise the action of heparin. Thrombin produced by the prothrombinase complex activates additional platelets resulting in release of platelet factor 4, which in turn inhibits the action of heparin (Lane et al., 1986). The clotting activity of thrombin on fibrinogen produces fibrin II monomer, which also has an inhibitory effect on heparin (Hogg and Jackson, 1989). Low molecular weight heparins also share some of these limitations.

In the venous thrombosis model, drug administration commences 30 min after the cotton threads are exposed to venous circulation. Thus unlike many other in vivo models of experimental thrombosis, the comparison of different agents are carried out under conditions of on going thrombosis. All agents tested were capable of inhibition of venous thrombosis. In addition to measuring antithrombotic efficacy, we evaluated several ex vivo clotting parameters as surrogate markers for assessing perturbation of hemostasis. As mentioned before, activated partial thromboplastin time was the most sensitive indicator of changes in heparin and C921-78 concentrations, whereas thrombin clotting time was closely correlated to changes in PPACK levels in plasma. The dose of each agent required to produce a near complete antithrombotic effect and the accompanying effect on clotting parameters clearly distinguished the direct fXa inhibitor from the other anticoagu-

lants in this study. Under conditions of venous thrombosis as measured in this in vivo model, doses of C921-78 which were fully antithrombotic did not cause significant changes in ex vivo clotting parameters. At comparable levels of antithrombotic efficacy, enoxaparin caused a twofold extension of activated partial thromboplastin times. The differential effect is even more pronounced for unfractionated heparin, in which case a sevenfold extension of ex vivo clotting accompanies antithrombotic doses. Evaluation of PPACK by similar criteria showed both extension of thrombin clotting time as well as concurrent thrombocytopenia (Hollenbach et al., 1994). Some of the effect on clotting parameters may be explained by the respective plasma levels of the different agents. When C921-78 is added to citrated rabbit plasma and the plasma evaluated in standard clotting assays, an inhibitor concentration of 840 nM is required for a twofold extension of activated partial thromboplastin time (Table 2). The corresponding concentration for twofold extension of prothrombin time is 7 μM. In anesthetized rabbits, a dose of 30 μg/kg + 0.9 μg/kg/min corresponds to a plasma drug concentration of 63 nM and thereby does not have significant effects on ex vivo clotting. For treatment of deep vein thrombosis in humans, a plasma level of 0.4–0.8 u/ml low molecular weight heparin is regarded as effective and safe (Bergqvist, 1996). In the deep vein thrombosis model, the plasma concentrations of rabbits dosed with the optimally antithrombotic doses of enoxaparin (200 u/kg + 2 u/kg/min) ranged between 3.8 and 4.7 u/ml. This is substantially higher than the commonly used dosing regimens and results in elevated activated partial thromboplastin times. Similarly the plasma level attained by fully effective doses of unfractionated heparin (100 u/kg + 2 u/kg/min), far exceed the recommended target therapeutic range of 0.3–0.7 u/ml for management of deep vein thrombosis and pulmonary embolism (Hirsh and Hoak, 1996). The high doses of both heparins required to exhibit optimal antithrombotic activity attest to the aggressive nature of venous thrombosis in this particular animal model. The capacity of C921-78 to be potently antithrombotic at doses that do not extend ex vivo clotting times, lends additional credence to the theory that inhibition of clot bound prothrombinase is an efficient way of achieving local inhibition of thrombosis without disrupting systemic hemostasis.

In the arterial thrombosis model, all agents had to be used in higher doses. In this model, intravenous infusion of drug is initiated prior to placing the thread in the arteriovenous shunt. Even under those conditions, none of the agents were able to completely inhibit thrombus growth at the highest tested dose. This data is in contrast to previously reported results for PPACK and for a specific fXa inhibitor. In a baboon model, PPACK is capable of interrupting platelet dependent thrombosis (Hanson and Harker, 1988). In another study of baboon arteriovenous shunts, tick anticoagulant peptide was capable of being fully antithrombotic at doses that extended basal activated partial thromboplastin time values less than twofold (Schaffer et al., 1991). It can be speculated that the activated platelet components of the clots in the arteriovenous model are not as susceptible to inhibitors of fXa or thrombin and to achieve complete cessation of thrombus growth an antiplatelet agent would be required. Historical data that supports this speculation bears on the efficacy of a synthetic pentasaccharide (truncated heparin capable of antithrombin III mediated inhibition of fXa) in a two component arteriovenous shunt in baboons (Cadroy et al., 1993). This fXa inhibitor was capable of inhibition of the fibrin rich venous type clot but was relatively ineffective on the platelet rich arterial clot. Anti-platelet agents have also been evaluated for their effectiveness against this type of arterial clots produced under high shear flow conditions. RGDV, a peptide that blocks platelet glycoprotein IIb IIIa activity, selectively blocks platelet dependent thrombus formation in this baboon model (Cadroy et al., 1989). The issue of relative antithrombotic efficacy in arterial versus venous thrombosis, was also noted for this antiplatelet agent. The doses of RGDV that were capable of inhibition of both platelet and fibrin deposition under high shear conditions, did not reduce fibrin accumulation under low shear conditions simulating venous blood flow.

One of the goals of this comparative study was to try to distinguish between the antithrombotic and the accompanying antihemostatic activity of each of the four agents. When an effective dose of C921-78 from the deep vein thrombosis model was studied for its effect on bleeding, the antithrombotic dose had little effect on cuticle bleeding time. Even when the dose escalation was taken to an eightfold higher level, the increase in bleeding time was not statistically significant. Enoxaparin, which is a dual inhibitor of thrombin and fXa, had significant increases during a similar dose escalation study. Unfractionated heparin, also a dual inhibitor but with equivalent inhibitory activity against these two enzymes, had significant increases in cuticle bleeding time at all doses tested. As mentioned before, PPACK at a fourfold higher dose had affected both bleeding time and platelet count of experimental animals.

Inhibition of thrombin activity, whether mediated through the action of antithrombin III on solution phase enzyme or by chloromethyl ketone on clot bound enzyme, increases bleeding time in experimental animals. These effects are significantly less when an inhibitor of thrombin generation is evaluated by the same criteria. Compared to fXa in solution, fXa as a part of the prothrombinase complex is 300,000-fold more efficient in thrombin generation (Mann et al., 1990). Thus prothrombinase on the surface of activated platelets rather than fXa in peripheral blood, is the true target of synthetic fXa inhibitors. C921-78 is inhibitory towards both solution phase fXa and membrane bound prothrombinase complexes (Betz et al., 1999). The reduced effect in prolongation of bleeding time and coagulation parameters may signify a greater benefit to

risk ratio for this agent. At doses several folds higher than that required for antithrombotic activity, C921-78 did not perturb beneficial hemostatic activity. This desirable profile of a selective and potent fXa inhibitor would be advantageous in therapeutic applications where excess bleeding is often the unwanted side effect of an anticoagulation regimen.

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