Comparative Effects of Two Direct and Indirect Factor Xa Inhibitors on Free and Clot-Bound Prothrombinase

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ABSTRACT
Factor Xa, as with thrombin, binds to the clot and contributes to the propensity of thrombi to activate the coagulation system. The aim of this work was to compare the extent of prothrombinase inhibition produced by two factor Xa inhibitors: the antithrombin III-dependent synthetic pentasaccharide (SR 90107/Org 31540) and DX-9065A, a direct factor Xa inhibitor. When incubated together with prothrombin, factor Xa, phospholipids, antithrombin III and calcium, clots formed from human plasma exhibited a prothrombinase activity as measured through fragment 1–2 (F1–2) generation. Ten washes of the clot were required to achieve complete removal of unbound factor Xa. The absence of F1–2 generation brought about by washed clots in buffer when factor V was omitted, or in the presence of annexin V, indicated that they contained bound factor Xa and phospholipids but no factor V/Va. In all tested experimental conditions, clot-bound-factor Xa-induced F1–2 generation was inhibited by SR 90107/AT and DX-9065A with IC50 in the same range of concentrations (0.5 μM). In contrast, the inhibition of prothrombinase formed with factor Xa, factor Va phospholipids and calcium in buffer was observed at significantly lower concentrations of DX-9065A than of SR 90107/AT (respective IC50 concentrations: 0.1 and 70 μM). In vivo, fibrin accretion onto a preformed thrombus as well as venous thrombosis induced in the jugular vein of rabbits was inhibited by SR 90107 and DX-9065A in the same range of concentrations therefore showing that inhibition of clot-bound factor Xa is a predominant factor for the antithrombotic activity of both direct and indirect inhibitors for factor Xa.

Thrombi are known to exhibit a procoagulant activity that is thought to play a fundamental role in the recurrence of thrombosis after thrombolysis and in the propagation of thrombosis. Binding of thrombin to clots is well documented. The fibrin binding sites have been localized and characterized (Vali et al., 1985; Weitz et al., 1990). It has been shown that thrombin bound to fibrin was protected from inactivation by macromolecular inhibitors and clot-bound thrombin because it was less efficient than free thrombin in proteolysing fibrinogen (Weitz et al., 1990; Hogg and Jackson, 1989). Recently, the characteristics of the binding of factor Xa to fibrin, fibrinogen and fibrinogen degradation products have been described and it was concluded that the affinity of factor Xa for these molecules was higher than that of thrombin (Iino et al., 1995). Moreover, it has been demonstrated that fibrin monomers did not affect the amidolytic activity of factor Xa and that fibrin-bound factor Xa could activate prothrombin therefore contributing to the procoagulant activity of thrombi (Eisenberg et al., 1993). Nevertheless, up to now, only few works dealing with the effect of inhibitors on clot-bound factor Xa are available. Some authors stated that prothrombinase activity due to clot-bound factor Xa was resistant to antithrombin III-dependent factor Xa inhibitors thrombi (Eisenberg et al., 1993), although several other reports indicated that the synthetic pentasaccharide, a selective factor Xa inhibitor with high affinity for AT, was an efficient compound to inhibit the growth of an experimental thrombus in several animal models (Cadroy et al., 1993; Carrié et al., 1994; Herbert et al., 1996).

Our aim was to study the prothrombinase activity of clot-bound factor Xa and to find out whether this activity was due to factor Xa alone or to an enzymatic complex formed in the clot. We therefore compared the effects of two factor Xa inhibitors with regard to the activity of clot-bound factor Xa or associated to phospholipid microvesicles. The factor Xa inhibitors studied were the synthetic pentasaccharide (SR 90107/Org 31540), which represents the minimal binding sequence of heparin to AT and which has been found to elicit a high and specific AT-mediated anti-factor Xa activity in vitro (Van Boeckel and Petitou, 1993), and DX-9065A, the first member of a newly developed family of synthetic and selective inhibitors of factor Xa (Hara et al., 1993, 1994, 1996; Katakura et al., 1993; Herbert et al., 1996). Moreover, we extended the results obtained in vitro with these compounds by determining their effects in vivo in a fibrin accretion model and in a jugular vein thrombosis model in rabbits.

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ABBREVIATIONS: AT, antithrombin-III; FPA, fibrinopeptide A; PPP, platelet poor plasma; PSPC, phospholipid vesicles; TBS, tris-buffered saline; PRP, platelet-rich plasma.
Materials and Methods

Materials. Human prothrombin, dioleoyl phosphatidyl serine, Gly-Pro-Arg-Pro, dioleoyl phosphatidyl choline and human factor V were from Sigma Chemical Co (Saint-Quentin-Fallavier, France). Phospholipid vesicles were made from a mixture of dioleoyl phosphatidyl choline and dioleoyl phosphatidyl serine. Vesicles composed of 20 molar percent dioleoyl phosphatidyl serine and 80 molar percent dioleoyl phosphatidyl choline were used throughout the experiments. TBS composition was 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4. Human annexin V was from Bender Med Systema (Vienna, Austria). Human α-thrombin (3000 IU/mg) was from Centre Regional de Transfusion Sanguine (Strasbourg, France). Thromboplastin (Thrombocel S) was from Behring (Marburg, Germany). Activated factor V (Va) was prepared by incubating 50 µl of human factor V (0.33 µM) with purified thrombin (0.25 U/ml) during 10 min at 37°C. Thrombin was then totally inactivated by 100 µl of hirudin (0.25 µg/ml). FPA and F1+2 concentrations were measured using an ELISA technique (FPA Asserachrom, Diagnostica Stago, Asnières, France) and Enzygnost F1+2 micro, (Behring, Marburg, Germany) respectively. SR 9107/Org 31540 (SR 9107), developed within a partnership agreement between Sanofi (Gentilly, France) and Organon (Oss, The Netherlands) was from Sanofi Recherche (Toulouse, France). DX-9065A was from Daichi Pharmaceuticals Co. Ltd. (Tokyo, Japan). Molecular weights of DX-9065A and SR 9107 were 571 and 1727 g, respectively. All other chemicals and solvents were reagent grade from Prolabo (Paris, France).

Preparation of plasma clots. Blood was collected from the antecubital vein of normal healthy volunteers using sodium citrate (9 vol blood/1 vol of 3.8% sodium citrate) as anticoagulant. After centrifugation for 15 min at 1500 × g the PPP was collected. Plasma clots were prepared from 900 µl citrated PPP by the addition of 100 µl CaCl2 (250 mM) and PSPC (20 µM) in TBS; from 900 µl of PRP or 900 µl of whole blood by the addition of 100 µl CaCl2 (250 mM) in TBS. Clots were formed around polystyrene hooks and incubated for 1 hr at 37°C under continuous shaking. Clots were then sequentially washed at room temperature with 2 ml of TBS to eliminate free thrombin, factor Xa, FPA and CaCl2 trapped within the clots. The washing procedure consisted of changing the washing buffer 10 times as follows: five times every 45 min, four times every hour and after overnight storage. Buffer was changed just prior to the assay.

Preparation of barium citrate-adsorbed plasma. Human plasma depleted of vitamin K-dependent factors was prepared by addition of 100 mM BaCl2 to pooled citrated PPP at 4°C for 60 min, followed by centrifugation to separate the precipitate. The supernatant was collected and dialyzed exhaustively against 0.15 M NaCl and 0.012 M sodium citrate, pH 6.0. The barium citrate adsorbed plasma (barium-adsorbed plasma) was stored as 1.0-ml aliquots at −70°C and thawed at 37°C immediately before use. The pH of the obtained plasma was 6.2

Characterization of clot-associated factor Xa activity. The activity of factor Xa associated to clots was characterized by measuring the extent of prothrombin activation. The activation of prothrombin was determined by measurement of changes in the concentration of FPA when the clots were incubated in barium-adsorbed plasma for 20 min at 37°C in the presence of 25 mM CaCl2 and 0.9 µM purified human prothrombin. The activation of prothrombin was also confirmed by measurement of changes in the concentration of prothrombin F1+2 when clots were incubated for 60 min at 37°C in TBS containing CaCl2 (25 mM) factor V (12.5 nM), PSPC (2 µM), AT-III (2.6 µM) and prothrombin (0.9 µM). Inhibition of clot-bound factor Xa by SR 9107 and DX-9065A was determined by incubating the clots in the presence of various concentrations of these compounds and IC50 values (concentrations that inhibited 50% of prothrombinase activity) were calculated using the four-parameter logistic model with a confidence interval of 95%. The adjustment was obtained by nonlinear regression using the Levenberg-Marquard algorithm in RS/1 software (BBN, Cambridge, MA).

Prothrombinase in solution. To determine the ability of the compounds to inhibit factor Xa included in the prothrombinase complex, the compounds were incubated for 5 min at 37°C in barium-adsorbed plasma containing factor Va (1.1 nM), PSPC (20 µM), factor Xa (9.2 pM) and CaCl2 (25 mM). Prothrombin activation was triggered by the addition of prewarmed prothrombin in the assay. After 15 min, FPA generation was measured. IC50 of prothrombinase inhibition by SR 9107 and DX-9065A were determined as described above.

Accretion of 125I-fibrinogen in an experimental thrombosis model in the rabbit. The antithrombotic activity of SR 9107 and DX-9065A was assessed by measuring their ability to inhibit the accretion of 125I-fibrinogen onto an autologous nonradioactive venous thrombi performed in the jugular veins of rabbits as described by Chiu et al. (1997). New Zealand male rabbits (2.7–3 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). Both jugular veins were exposed and a 2-cm segment of each vein was isolated. Each segment was emptied of blood and clamped. One ml of blood was collected from a carotid artery and mixed with 50 µl of thrombin (20 U/ml). Clotting blood (150 µl) was immediately injected into both isolated segments. Blood flow was restored 2 min. later. A 10-cm length silk thread was passed longitudinally through the forming thrombus and the vessel wall to keep the thrombus in place. Fifteen min after the thrombus was formed, each animal was injected with 20 µCi 125I-labeled human fibrinogen. The animals were treated with saline or the indicated dose of SR 9107 or DX-9065A as an infusion for 4 h. At the end of the infusion period, both venous segments containing the thrombi were tied off, slit open longitudinally and the remaining thrombi were removed. The radioactivity of the thrombus was used as a marker of thrombus growth. The results were expressed as the percent reduction of the radioactivity of the thrombus in SR 9107- or DX-9065A-treated rabbits in comparison with the animals receiving saline.

Stasis-induced venous thrombosis in the rabbit. Rabbits were anesthetized by an i.v. injection of sodium pentobarbital (30 mg/kg, i.v.). Stasis-induced venous thrombosis was induced according to Buchanan et al. (1985) with slight modifications. Each jugular vein was isolated and two loose sutures were placed 2 cm apart. Test compounds or placebo were administered i.v. through a marginal ear vein 5 min before ligation of the jugular veins. Recombinant human tissue factor (1 ng/kg) was injected 5 min before the induction of stasis. Both jugular vein segments were occluded by the distal and proximal sutures and stasis was maintained for 15 min. The veins were opened longitudinally, and the thrombus, if apparent, was removed, blotted on filter paper and weighed. Wet weights of thrombi were averaged for left and right jugular veins. Test compounds or the vehicle were administered i.v. 5 min before the i.v. injection of tissue factor.

Statistical evaluation. The results shown are arithmetic means ± S.E.M. Grouped data were analyzed for significance using the Kruskal-Wallis nonparametric analysis of variance taking P < .05 to indicate a significant difference. IC50 values were determined using the four-parameter logistic model with a confidence interval of 95%. The adjustment was obtained by nonlinear regression using the Levenberg-Marquard algorithm in RS/1 software (BBN). The protocol of this study has been approved by the animal care and use committee of Sanofi Recherche.

Results

Determination of the activity of clot-bound factor Xa. To determine the activity of factor Xa associated with the clot, washed clots were incubated in human plasma that was depleted of vitamin K-dependent enzymes (barium-adsorbed plasma) and repleted with prothrombin (0.9 µM). The factor
Xa activity was characterized by the generation of FPA at 37°C. As shown in figure 1A, a rapid and marked increase in the concentration of FPA occurred between 10 and 20 min of incubation resulting in a FPA concentration of 1480 ± 680 nM at 20 min. This FPA formation could not be attributable to any prothrombinase contamination because no FPA formation occurred when recalcified repleted barium-adsorbed plasma was incubated in the absence of clots or in the absence of prothrombin.

Although the pH of barium-adsorbed plasma was below the physiological value (6.2), these results were in agreement with those obtained by Eisenberg et al. (1993). However, when the pH was adjusted to 7.4, determination of FPA generation cannot be determined due to the rapid coagulation of the plasma. For that reason, we determined clot-bound factor Xa activity in buffer at pH 7.4, containing CaCl₂ (25 mM), factor V (12.5 nM), PSPC (2 μM), AT III (2.6 μM) and prothrombin (0.9 μM). As shown in figure 1B, clot-induced generation of F₁₋₂ in buffer was compared to clot-induced generation of F₁₋₂ in barium-adsorbed plasma. Although, up to 30 min there was no significant difference in F₁₋₂ generation between both conditions, at 60 min, however, F₁₋₂ concentrations were higher in buffer than in plasma. To validate the washing procedure, incubation of clot in buffer and determination of F₁₋₂ concentration was chosen. The washing procedure consisted in changing the washing buffer 10 times as follows: five times every 45 min, four times every hour and after overnight storage. Buffer was then changed just before the assay. To monitor removal of factor Xa from clots, factor Xa activity of clots was measured after 3, 5 and 10 washes. In parallel, washing buffers were mixed with CaCl₂ (25 mM), factor V (12.5 nM), PSPC (2 μM) and prothrombin (0.9 μM), and F₁₋₂ generation was measured. Under these experimental conditions, a strong decrease in clot-induced F₁₋₂ formation was observed between the fifth and the tenth wash (fig. 2). Free factor Xa present in the third and fifth washing buffers induced the generation of a significant amount of F₁₋₂, although the factor Xa-induced generation of F₁₋₂ remaining in the tenth wash was considered as negligible showing that, under these experimental conditions, almost all of the prothrombinase activity of the clots was attributable to clot-bound factor Xa. Moreover, it is
noteworthy that, under the same washing conditions, no significant differences were observed between $F_{1+2}$ generation induced by a clot formed from PPP and supplemented with 2 $\mu$M of PSPC, formed from PRP or formed from whole blood ($200 \pm 16$, $201 \pm 15$ and $205 \pm 20$ nM, respectively; mean $\pm$ S.E.M, $n = 4$) therefore showing that, whatever the method of clot formation (PPP, PRP or whole blood) the amount of clot-bound factor Xa was equivalent.

Characterization of the components of the prothrombinase bound to the clot. To determine if a phospholipid surface and factor Va were involved in the prothrombinase activity of the clots, clots were incubated in Ca$^{+}$ buffer and 1) in the presence of factor V and PSPC, 2) in the presence of factor V without PSPC, 3) in the absence of factor V and PSPC, 4) in the presence of factor V and annexin V that prevents the association of the coagulation enzymes to anionic phospholipids. The results shown in figure 3 demonstrate that PSPC added during the incubation period was not necessary for the prothrombinase activity of the clots because there were no significant differences in concentration of $F_{1+2}$ generated in the presence or in the absence of PSPC. However, depletion of factor V significantly reduced the generation of $F_{1+2}$. In the presence of annexin V, clot-induced $F_{1+2}$ generation was strongly reduced (87%) showing that “endogenous” phospholipids necessary for the clot-bound prothrombinase were trapped in the clots. Taken together, these findings allowed us to conclude that the prothrombinase activity exhibited by clots was due to bound factor Xa, factor Va, clot-trapped phospholipids and Ca$^{+}$ whose absence prevented any activity in all cases (data not shown). Clot-bound prothrombinase components are therefore assumed to be similar to the prothrombinase complex formed onto microvesicles. The inhibitory effects of two factor Xa inhibitors, SR 90107 and DX-9065A, were therefore compared in these two environments.

Effect of inhibitors on clot-bound factor Xa. The ability of SR 90107 and DX-9065A to inhibit clot-bound factor Xa was studied in barium-adsorbed plasma. The results shown in figures 4A and B indicate that both DX-9065A and SR 90107 inhibited clot-induced prothrombin activation with almost identical concentration response curves. Calculated IC$_{50}$ values for SR 90107 and DX-9065A were 0.096 [(0.06–0.16) $\mu$M] and 0.055 (0.007–0.125) $\mu$M (FPA) and 0.58 (0.12–2.7) $\mu$M and 0.44 (0.14–1.17) $\mu$M ($F_{1+2}$). Response with clots...
without compounds (200 ± 16 nM) was considered as 100% and buffer without clot (10 ± 0.5 nM) was 0%. In both cases, there were no significant differences between IC_{50} values.

Moreover, the effect of both inhibitors on clot-bound factor Xa was evaluated by incubating the clot in buffer as described in “Materials and Methods.” As shown in figure 4C, under these conditions, a concentration-dependent inhibition of the F_{1,2} generation was observed with IC_{50} values of 0.54 (0.34–0.8) μM and 0.50 (0.05–0.9) μM for DX-9065A and SR 90107, respectively.

**Inhibition of factor Xa included in the prothrombinase complex onto microvesicles.** To compare the inhibitory effects of SR 90107 and DX-9065A on factor Xa bound onto microvesicles, prothrombinase complex was formed with Ca^{2+}, PSC, factor Xa and factor Va. This complex added to barium-adsorbed plasma repleted with prothrombin led to the generation of FPA (250 ± 20 nM). To verify that the FPA generation was due to the complex, we first determined that, in the absence of one of the four elements of this complex, no FPA generation was observed (data not shown). The results in figure 5 indicate that when various concentrations of the two factor Xa inhibitors were added to the incubation medium in the presence of the prothrombinase complex, a concentration-dependent inhibition of FPA generation was observed with DX-9065A [IC_{50} value of 0.08 (0.02–0.2) μM] whereas SR 90107 inhibited factor Xa included onto microvesicles only at the highest concentration used.

**Effect of the compounds on the accretion of fibrinogen in vivo.** Because the results obtained in vitro revealed an inhibitory activity of SR 90107 and DX-9065A regarding clot-bound factor Xa, the effect of these two compounds was determined in an in vivo model of fibrinogen accretion in the rabbit. This model allowed us to determine the ability of the clots to generate in situ fibrin from fibrinogen. The effects of SR 90107 and DX-9065A on inhibition of 125I-fibrinogen accretion onto the preformed thrombi are shown in figure 6. In saline-treated animals, 5.5 ± 0.8 μg (n = 9) of 125I-fibrinogen was accreted onto the preformed thrombus after 4 hr. SR 90107 and DX-9065A revealed a dose-dependent inhibition of fibrinogen accretion. The ED_{50} (dose that inhibits 50% of 125I-fibrinogen accretion) was of 512 ± 10 and 130 ± 20 μg/kg for SR 90107 and DX-9065A, respectively.

**Stasis-induced thrombosis after injection of tissue factor in the rabbit.** Using a combination of a thrombogenic challenge (1.0 ng/kg of tissue factor) and stasis, DX-9065A and SR 90107 were tested for their ability to affect thrombus formation in a venous thrombosis model in the rabbit. DX-9065A and SR 90107 administered s.c., 1 hr before thrombosis induction displayed a significant, dose-dependent anti-thrombotic effect (fig. 7). The ED_{50} values were 51 ± 7 and 60 ± 1 μg/kg (n = 10) for DX-9065A and SR 90107, respectively.

**Discussion**

It has been suggested that the propensity of thrombi to induce activation of the coagulation system plays an important role in the recurrence of thrombosis after thrombolysis and in the propagation of thrombi. One potential contributor of the procoagulant activity of thrombi is the persistence of activity of thrombin bound to fibrin (Wilner et al., 1981; Francis et al., 1983; Weitz et al., 1990). In addition to throm-
bin, factor Xa formed in vivo contributes to the procoagulant properties of thrombi in an important way and has been shown to be a major determinant of the procoagulant activity of whole clots and arterial thrombi (Eisenberg et al., 1993). The results of this study confirm that factor Xa binds to the clot and remains enzymatically active and show that, despite the presence of physiologic concentrations of antiproteases, clot-bound factor Xa can still cleave prothrombin as shown by FPA and F112 generation in vitro. Levels of FPA and F112 were therefore used as an index of unopposed factor Xa activity. In our model system, the time course of FPA and F112 generation by clot-bound factor Xa was different from that produced by the fluid-phase enzyme. In the presence of a clot that has been thoroughly washed to remove trapped FPA, F112 and unbound factor Xa, there was a progressive FPA and F112 generation throughout the incubation period. Clots of increasing size generated more FPA and F112 presumably because the larger area exposed more factor Xa to prothrombin. In this respect, it should be pointed out that washing of the clot during the experimental procedure is a key step for the appropriate study of the characteristics of clot-bound factor Xa. Indeed, we have established that up to 10 washes were required to achieve the complete removal of unbound factor Xa from the clots. Under these experimental conditions, when nonspecific protein trapping was negligible, we found that, in buffer, clot-induced thrombin generation did not vary whether the clots were prepared from platelet poor plasma, platelet rich plasma or whole blood therefore showing that, under our experimental conditions, blood cells play a negligible role in the generation of thrombin by factor Xa at the surface of clots. This observation also shows that the capacity of the fibrin network to bind factor Xa was equivalent when the clots were formed from plasma, platelets or whole blood. A direct interaction of factor Xa with fibrin has already been suggested by Eisenberg et al., 1993 and is consistent with the observation that fibrin II monomer attenuates antithrombin III-mediated inhibition of factor Xa (Hogg and Jackson, 1989). Moreover, a recent study showed that there is indeed a specific binding site for factor Xa on fibrinogen which is located in the boundary between the central E domain and the terminal D domain of fibrinogen and is apparently distinct from the reported thrombin binding site (Iino et al., 1995). Another finding of this study was that both phospholipids and factor Va appeared to be involved in the conversion of prothrombin to thrombin by clot-bound factor Xa. Although factor Va could result from the activation of factor V by either newly generated or clot-bound thrombin, the source of phospholipids however, is still unknown but we showed that they might be trapped in the clot during its formation. Indeed, although the addition of exogenous phospholipids to the clot after its formation did not result in an increase of F112 generation, the addition of annexin V abolished the activity of clot-bound prothrombinase. Therefore, because Ca++, phospholipids and factor Va are required for prothrombin activation, this prothrombinase complex appears to be analogous to the prothrombinase complex formed onto microvesicles or on platelets (Rosing et al., 1980). Moreover, although we were unable to demonstrate significant factor VIIa or factor IXa activity associated with the clot formed in vitro, we cannot exclude the possibility that the activity of factor VIIa/tissue factor or factor IXa/factor VIIIa complexes contributes to clot-induced procoagulant activity in vitro.

To further clarify the potential importance of factor Xa bound to fibrin, relative to free factor Xa, we have evaluated the activity of two selective factor Xa inhibitors exhibiting different mechanisms of action: SR 90107 is a pentasaccharide that shows high affinity for AT and behaves as a potent and selective catalyst of the anti-factor Xa activity of this serpin (Van Boeckel and Petiotou, 1993). DX-9065A is a synthetic compound that potently inhibits factor Xa directly at its catalytic site (Har et al., 1993, 1994, 1995; Katakur et al., 1993; Herbert et al., 1996). Recent studies with these compounds in animals indicate that inhibition of factor Xa is effective in attenuating thrombosis in response to arterial injury, venous stasis and recurrent thrombosis after thrombolysis (Cadroy et al., 1993; Carrié et al., 1994). In the course of these studies, we and several authors compared the activity of both types of inhibitors and found that they differentially inhibited thrombosis depending on the experimental model used. Upon the various hypothesis raised, it was suggested that such differences in activity observed between these two types of inhibitors might be due to a differential inhibition of clot-bound compared to fluid-phase factor Xa (Herbert et al., 1996; Sitko et al., 1992; Prager et al., 1994). Judging from the results of our study, our data are not consistent with these findings and indicate that AT-dependent inhibitors may be as effective as direct factor Xa inhibitors in inhibiting phospholipid/factor Va-bound factor Xa (in solution or on blood cells). Therefore, unlike that observed in the case of thrombin, clot-bound factor Xa appears to be equally sensitive to both types of inhibitors therefore showing that steric hindrance of the AT/SR 90107 complex does

**Fig. 7.** Effect on stasis-induced venous thrombosis in the rabbit. DX-9065A or SR 90107 were administered i.v. 1 hr before the induction of stasis performed in combination with tissue factor (1 ng/kg i.v.). Venous thrombosis was induced as described in "Materials and Methods." Each point is the mean of 10 animals/group. Kruskal-Wallis test was used to determine the difference between vehicle- and drug-treated groups: *P < .05; **P < .01; ***P < .001.
not affect its inhibitory capacity as observed with regard to thrombin for the AT/heparin complex (Weitz et al., 1990). These results are therefore different from that described by Eisenberg et al. who showed that the prothrombinase activity of whole blood clots was resistant to inhibition by AT-dependent inhibitors (Eisenberg et al., 1993). However, a major experimental difference can explain such a discrepancy. Indeed, because we found that both types of inhibitors behaved differently regarding the activity of fluid-phase factor Xa, we took great care to decrease the nonspecific protein trapping in the clot and in particular to decrease the level of free factor Xa present in the clot less than 4 to 5% of the total factor Xa present in the preparation. To reach this goal, at least 10 washes of the clot were needed. Because, in their system, Eisenberg et al., (1993) washed the clots three times only (in these conditions, free factor Xa present in the clot represented more than 45% of the total prothrombinase activity of the clot) and a high level of non-specific factor Xa binding might explain the differences observed between both results.

These findings have important therapeutic implications because the presence of a high level of clot-bound factor Xa activity suggest that inhibition of clot-associated procoagulant by thrombin-specific inhibitors will not be effective enough to prevent continued activation of prothrombin whereas inhibition of clot-bound factor Xa by either direct or indirect inhibitors may attenuate clot-associated procoagulant activity. Recent results demonstrating the efficacy of tick anticoagulant peptide (Sitko et al., 1992), SR 90107 (Bernat et al., 1996) or DX-9065A (Herbert et al., 1996) in preventing reocclusion after thrombolysis are consistent with this hypothesis.

Thus, although there is evident similarities between thrombin and factor Xa as key factors for the development of thrombosis, the studies presented here performed in cell-free systems show that they behave differently to what concerns the potential of inhibitors to modulate their activity when bound to clots. Although these results raise a discrepancy with previous data (Eisenberg et al., 1993), they are consistent with all the data obtained in vivo in several experimental models showing that both direct and indirect factor Xa inhibitors are potent antithrombotic agents. Accordingly, the results of this study reinforce the potential for the use of either direct or indirect inhibitors of factor Xa as promising therapeutic agents.

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