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Inhibition of Purified Factor Xa Amidolytic Activity May Not Be Predictive of In Vivo Thrombosis

Implications for Identification of Therapeutically Active Inhibitors

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Objective—In this study we test the hypothesis that blood/plasma-based prothrombinase assays, rather than inhibition of purified factor Xa (fXa), are predictive of in vivo antithrombotic activity.

Methods and Results—Six fXa inhibitors with equivalent nanomolar K_i were studied in thrombin generation assays using human plasma/blood and endogenous macromolecular substrate. In all assays, benzamidine inhibitors were more potent (100 to 800 nmol/L) than the aminoisoquinolines (5 to 58 $\mu\text{mol/L}$) or neutral inhibitors (3 to 10 $\mu\text{mol/L}$). A similar rank order of compound inhibition was also seen in purified prothrombinase assays as well as in a rabbit model of deep vein thrombosis.

Conclusions—Assays using prothrombinase with protein substrates are better predictors of in vivo efficacy than fXa K_i using amidolytic substrates. (*Arterioscler Thromb Vasc Biol.* 2003;23:1098-1104.)

Key Words: factor Xa ■ prothrombinase ■ coagulation inhibitors

Factor Xa (fXa) has long been viewed as a target for anticoagulation therapy because it is at the intersection of the intrinsic and extrinsic coagulation pathways, and its selective inhibition is presumed to have a major anticoagulatory effect. The prothrombinase complex, of which fXa is the enzymatic component, is assembled on the surface of activated platelets, and inhibition of this localized activity of fXa may provide superior efficacy without systemic bleeding consequences. Specific inhibition of fXa by agents such as Fondaparinux has validated its role as a target for antithrombotic drug development.¹ Anticoagulants that target fXa are not specific, active site inhibitors. During their course of action, injectable anticoagulants like heparin use physiological fXa inhibitors such as the serpin antithrombin III. The orally bioavailable agent warfarin functions as a nonspecific anticoagulant by reducing plasma levels of several vitamin K–dependent coagulation factors, fXa being one of several proteins that are affected. The major goal for the anti-fXa small molecule drug development effort is to find pharmaceutically acceptable agents that can mimic the efficacy of warfarin without its accompanying safety problems.² Although several secondary screens and animal models are used to optimize structure activity relationships (SARs) of potential fXa inhibitors, the initial evaluation is focused on active site-directed serine protease inhibitors that are generally identified by inhibition of purified human fXa.³ However, as mentioned before, the true target for these potential drugs is

the prothrombinase complex. Unless it is incorporated in the complex, fXa does not cleave prothrombin at a rate that is sufficient to support hemostasis.⁴ Because of technical difficulties associated with reconstituted prothrombinase as a screening tool, fXa screens are frequently used to select inhibitors for additional study. We wanted to test the hypothesis that inhibition of fXa alone is a sufficient predictor of a compound's capacity for inhibition of in vivo thrombosis. For this purpose, we chose 6 specific small molecule fXa inhibitors of comparable potency (similar K_i) as determined by purified fXa screens. The inhibitors belong to 3 different chemical classes and were evaluated in reconstituted and in vitro plasma/blood-based prothrombinase assays. Inhibitor efficacy was also tested in an in vivo model of venous thrombosis. In the present study, we show results attesting to the fact that potency of inhibition of fXa is not necessarily representative of the inhibitory capacity of active site-directed agents in settings of in vivo thrombosis.

Methods

Kinetics of Enzyme Inhibition

Human fXa, fIXa, fVIIa, factor Va (fVa), prothrombin 2, and α thrombin were from Hematologic Technologies. Half-maximal inhibition of thrombin, factor IXa (fIXa), and factor VIIa (fVIIa)/tissue factor (TF) activity were measured as described.^{5–7}

K_i Determination

fXa (1 nmol/L) was added to a 96-well plate containing inhibitor (5 pmol/L to 3 $\mu\text{mol/L}$) in buffer A (20 mmol/L HEPES, 0.15 mol/L

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NaCl, 0.1% PEG-8000, 5 mmol/L CaCl₂, pH 7.4). S-2765 (N- α -Z-D-Arg-Gly-Arg-p Nitroanilide, Diapharma) at 200 μ mol/L was added, and substrate hydrolysis was monitored at 405 nm. Data were analyzed by Batch Ki software (BioKin).

k_{off} Determination

Inhibitor (200 nmol/L) was preincubated with fXa (100 nmol/L) for 30 minutes. The complex was diluted in 1 mmol/L Spectrozyme FXa (MeO-CO-D-Chg-Gly-Arg-p Nitroanilide, American Diagnostica), and recovery of enzymatic activity was monitored at 405 nm. k_{off} was calculated as in the study by Betz et al.⁸

k_{on} Determination

Equal volumes of fXa (100 nmol/L) and substrate plus inhibitor (400 and 2 μ mol/L) were added to each reservoir syringe of a stopped-flow reaction analyzer (Applied Photophysics). Change in absorbance at 405 nm was monitored after the mixing of syringe contents. The stopped-flow traces were analyzed as in the study by Betz et al.⁸

Protein Binding

The extent of rabbit plasma protein binding was determined by the ultra filtration method described in the study by Wright et al.⁹

Blood Collection

Blood from healthy volunteers (a minimum of 3 donors) was drawn and processed to produce a plasma pool as described in the study by Hemker et al.¹⁰ Plasma aliquots were stored at -80°C. The platelet-rich plasma (PRP), prepared at the time of the experiment, was the supernatant removed from a 900g centrifugation of citrated whole blood.

Thrombin Generation in Reconstituted Human Prothrombinase Complex

Inhibition assays were carried out as reported.¹¹ FXa was used at a concentration of 1 nmol/L, and after 10 minutes of incubation, the reaction was quenched by addition of soybean trypsin inhibitor (0.5 mg/mL, Sigma).

Thrombin Generation in Human Plasma

The protocol was adapted from the method used by Hemker et al.¹⁰ Reptilase-treated plasma pool was added to DMSO-solubilized fXa inhibitor in 96-well plates, followed by substrate Pefachrome TG (Pefa-5134, Pentapharm) at a final concentration of 500 μ mol/L and CaCl₂ at a final concentration of 16.7 mmol/L. Thrombin generation reactions were started by addition of 640 pmol/L TF (Innovin, Dade Behring), and absorbance was monitored at 405 nm at 37°C for 22 minutes.

Thrombin Generation in Platelet-Rich Plasma

The assay was modified from that reported by Hemker et al.¹² PRP was treated with Pefabloc FG (Pefa-6003, Pentapharm) at 2.3 mg/mL at 37°C for 10 minutes in Sigmacoted (Sigma Aldrich) aggregometer tubes (Chronolog). Additions of 20 mmol/L CaCl₂, 2 μ mol/L TRAP (a thrombin receptor agonist peptide, Ser [pFPhe]-Har-Leu-Har-Lys-NH₂¹³), 500 μ mol/L substrate Z-Gly-Gly-Arg AMC (Bachem), and inhibitor in DMSO at various concentrations were added to the tubes. Thrombin generation was triggered by addition of 0.8 pmol/L TF and was monitored for 25 minutes at 37°C using 360 nm excitation and 437 nm emission in a modified luminescence spectrometer (SLM-Aminco).

Thrombin Generation in Whole Blood

FXa inhibitor was added to blood within 2 minutes of blood collection. The blood was stirred on a Multi-magnestir plate (Laboratory Line Instruments) at room temperature. Aliquots withdrawn at specific time points were treated with a quench solution.¹⁴ Enzyme immunoassay for the determination of human thrombin-antithrombin III complex (TAT) was done according to manufacturer's instruc-

tions (Enzygnost TAT micro, Dade Behring). Quantitation of fibrinopeptide A (FPA) was by LC/MS/MS.

Rabbit Deep Vein Thrombosis Model

The experimental protocol has been described.¹⁵ Briefly, weight of a thrombus formed on cotton threads introduced in the abdominal vena cava of an anesthetized rabbit was measured with and without administration of inhibitor. In this study, a modification of the published protocol was used in that the thrombogenic device was introduced simultaneously with the initiation of drug administration rather than 30 minutes before drug infusion. Blood samples obtained during the experiment were monitored for inhibitor plasma concentration and coagulation parameters. Prothrombin times (PTs) were evaluated using Thromboplastin C Plus (Dade) on an automated coagulation timer (ACL 3000, Instrumentation Laboratory). Quantitation of plasma concentration of inhibitors was carried out by LC/MS/MS. Except where noted, 6 animals per group were used for each inhibitor tested and each group had its own control set of animals (n=6). Our protocol was approved by the Institutional Animal Care and Use Committee at Millennium Pharmaceutical Inc and was in compliance with United States Drug Association federal guidelines (*Guide for the Care and Use of Laboratory Animals*).

Results

Inhibition of Purified Human fXa

A series of fXa inhibitors from different chemical classes have been designed to improve their pharmacokinetic

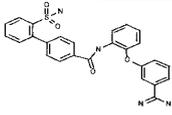
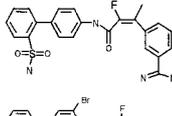
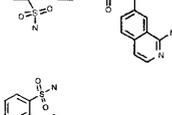
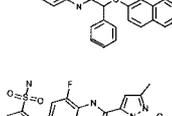
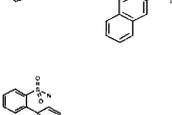
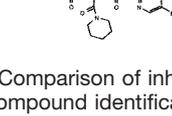
Compound ID	Chemical Structure	Thrombin IC ₅₀ (μ mol/L)	fXa Ki (nmol/L)	k _{on} (μ M ⁻¹ S ⁻¹)	k _{off} (S ⁻¹)	Prot bind (%)
C2092-28		>10	1.8	27	0.100	82.7
C1942-36		3.2	1.1	23	0.025	80.4
C2072-24		4.9	1.3	23	0.059	99.8
C1924-81		>10	1.2	17	0.059	99.9
C2112-42		>10	1.4	53	0.055	97.2
C2104-75		>10	1.4	27	0.024	96.1

Figure 1. Comparison of inhibitory activity in purified enzyme assays. Compound identification and chemical structure of the 6 compounds studied are shown. The compounds represent 3 chemical classes of inhibitors (P1 benzamides, aminoisoquinolines and neutrals), and the fXa K_is are similar. fXa specificity is demonstrated by lack of inhibition against thrombin. Additionally, all compounds have similar values for k_{on} and k_{off}. Extent of rabbit plasma protein binding is similar for the benzamides and higher for the aminoisoquinolines.

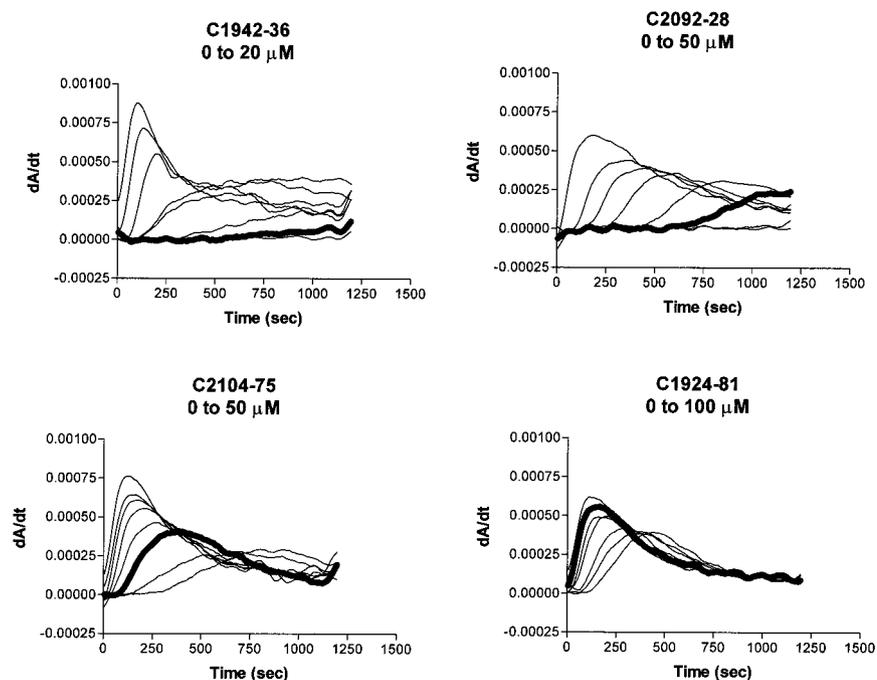


Figure 2. Tissue factor–mediated thrombin generation in human plasma. Each of the 4 panels depicts a dose-response curve for inhibition of the rate of thrombin generation (dA/dt) over the time course of experimentation. Each inhibitor was independently tested 4 to 6 times. Representative curves are shown here. The range of concentration evaluated for each inhibitor is as follows: C1942-36 (8 concentrations between 0 and 20 $\mu\text{mol/L}$, bolded at 10 $\mu\text{mol/L}$); C2092-28 (8 concentrations between 0 and 50 $\mu\text{mol/L}$, bolded at 12.5 $\mu\text{mol/L}$); C2104-75 (8 concentrations between 0 and 50 $\mu\text{mol/L}$, bolded at 10 $\mu\text{mol/L}$); and C1924-81 (8 concentrations between 0 and 100 $\mu\text{mol/L}$, bolded at 10 $\mu\text{mol/L}$). The thrombin lag 2X extension is calculated by determining the concentration of inhibitor required to extend the time of peak thrombin generation by 2-fold.

properties. We have selected 6 of these inhibitors for our comparative study.^{16,17,18} C2092-28 and C1942-36 belong to the P1 benzamidines series, C2072-24 and C1924-81 are in the class of P1 aminoisoquinolines, and C2112-42 and C2104-75 contain neutral moieties in both the P1 and P4 position. As shown in Figure 1, all of the chosen compounds were capable of inhibition of fXa at low nanomolar concentrations and, when assayed in the same concentration range, did not cross-react with the related serine protease thrombin. Representative inhibitors from the 3 classes (C2092-28, C1942-36, C1924-81, and C2104-75) were also evaluated for inhibitory activity against amidolytic substrate cleavage by fVIIa/TF and by fIXa. For each of the 4 compounds assayed to a concentration of 1 $\mu\text{mol/L}$, no inhibitory activity was detected against either fVIIa or fIXa (data not shown). Stopped-flow measurements of k_{on} and k_{off} showed that the compounds share similar modes of inhibition against tripeptide substrate cleavage by solution phase fXa. Compared with previously reported transition state analog peptide inhibitors of fXa, all 6 compounds would be classified as reversible inhibitors.^{3,8} Because our *in vivo* and some of our *in vitro* evaluations involve the presence of blood components, we measured the extent of plasma protein binding by these agents and found protein binding for the 2 benzamidines was significantly lower than that for the neutral and aminoisoquinoline inhibitors (Figure 1). To investigate the effect of variable protein binding capacity on the potency of compounds, K_i determinations for fXa inhibition were carried out in buffer A containing 1 mg/mL BSA. The observed K_i for C2104-75 (a 96% protein-bound inhibitor) as well as those for C2092-28 and C1942-36 (80% to 83% protein-bound inhibitor) did not change in the presence of added protein in the assay media (data not shown).

Tissue Factor–Mediated Thrombin Generation in Human Plasma

We decided to use a human plasma-based system to compare the relative potency of fXa inhibitors during cleavage of the physiological substrate prothrombin. The time course of thrombin generation was measured by following the kinetics of cleavage of a specific paranitroanilide substrate. The derivative of the optical density measurement is proportional to the sum of activities derived from free thrombin and thrombin- α_2 macroglobulin complex. Figure 2 shows the concentration-dependent extension of lag of TF-mediated thrombin generation. The bolded rates of thrombin generation correspond to approximately equivalent amount (10 to 12.5 $\mu\text{mol/L}$) of each of the fXa inhibitors. The concentration required for 2-fold extension of time required for peak thrombin generation can be used for a relative ranking of the 6 compounds. As shown in Table 1, the superior potency observed for C1942-36 and C2092-28 translates into much lower effective inhibitory concentrations (400 and 800 nmol/L, respectively) than those for C2104-75 and C1924-81 (5 and 47 $\mu\text{mol/L}$, respectively). In general, the potency of the P1 benzamidines was at least 7-fold superior to the other 2 classes of compounds.

Thrombin Generation in Platelet-Rich Plasma

The interactions between activating/aggregating platelets and components of the coagulation cascade are of importance during thrombotic events. Because an active site-directed fXa inhibitor should be capable of inhibition of prothrombinase complexes on the surface of activated platelets, we decided to use thrombin generation in PRP to rank the inhibitors from different classes. Two P1 benzamidines, 1 P1 aminoisoquinoline, and 1 P1 neutral inhibitor were used for this purpose. The continuous determination of thrombin generation and the first derivative (rate of generation) for selected inhibitor

TABLE 1. Correlation of In Vitro and In Vivo Parameters

Compound	FXa Ki, nmol/L	Thrombin Lag 2X Extension, nmol/L	Thrombin Generation in PRP IC ₅₀ , nmol/L	Whole Blood Thrombin Generation	In Vivo Model
C2092-28	1.8	800	214	Inhibitory at 500 nmol/L	Inhibitory at 0.8 μ mol/L*
C1942-36	1.1	400	106	Inhibitory at 200 nmol/L	Inhibitory at 0.2 μ mol/L*
C2072-24	1.3	5000	No inhibition at 0.2 μ mol/L and higher†
C1924-81	1.2	47 000	58 000	Partial inhibition at 20 μ mol/L	No inhibition at 0.7 μ mol/L
C2112-42	1.4	2800	No inhibition at 1.3 μ mol/L
C2104-75	1.4	5000	3100	Inhibitory at 10 μ mol/L	No inhibition at 7 μ mol/L

All six compounds studied have similar Ki values to fXa. Prothrombinase inhibition in plasma, expressed as an extension in time to maximum thrombin generation, was 5- to 10-fold better with benzamidines than with the other classes of compounds. Thrombin generation in PRP was inhibited by the benzamidines at nanomolar concentrations, but micromolar amounts were required by the aminoisoquinoline or the neutral compound tested. Whole blood assays showed inhibition of thrombin generation markers that required concentrations of inhibitor 10 to 20 times that of the aminoisoquinoline or neutral over the benzamidine. Thrombus inhibition in an in vivo model of thrombosis was only demonstrated by the benzamidines.

*Significant inhibition of thrombosis compared with vehicle controls ($P < 0.05$, Student's *t* test).

†C2072-24 was tested at two doses, 0.8 and 2.4 mg/kg. Technical difficulties with LC/MS/MS protocol prevented us from accurately determining rabbit plasma concentrations at the higher dose.

concentrations are shown in Figure 3. All 4 compounds tested were inhibitory in a dose-responsive manner but with greatly differing potencies. The 2 P1 benzamidines show a 50% decrease of fluorescence signal from the averaged control at approximately 100 nmol/L. The P1 neutral inhibitor (C2104-75) can achieve comparable inhibition but at a 5 times higher concentration. The P1 aminoisoquinoline fared the worst and needed greater than 50 times the amount for half-maximal inhibition. The fluorescence titration data were used to calculate the amount of inhibitor required for half-maximal inhibition of area under the curve (Table 1).

Thrombin Generation in Whole Blood

To investigate inhibitory capacity of the inhibitors in the presence of all the components of whole blood, we monitored 2 markers of thrombin generation in freshly drawn, nonanticoagulated blood. Unlike the 2 previously described systems, the initiation of clotting was not carried out by addition of exogenous TF but by activation of platelets by gentle magnetic stirring.¹⁴ This experimental protocol produces micromolar amounts of FPA, which was subsequently degraded during the 60-minute observation period (Figure 4). TAT, the other marker of thrombin generation, attained peak levels after a slightly longer lag period and remained relatively stable during sampling. Figure 4 shows a representative

experiment comparing 2 P1 benzamidines, 1 P1 neutral and 1 P1 aminoisoquinoline. To achieve comparable potency of inhibition, the agents had to be evaluated at varying doses with C1942-36 and C2092-28 tested between the concentrations of 200 nmol/L and 1 μ mol/L and C2104-75 tested at 10 μ mol/L. However C1924-81 did not attain similar inhibitory activity at the highest dose tested (20 μ mol/L) (Figure 4 and Table 1).

Thrombin Generation in Reconstituted Prothrombinase Complex

Data for inhibition of thrombin generated by a purified, reconstituted human prothrombinase complex is shown in Table 2. The rank order of inhibition by the studied compounds is similar to that seen in the 3 human plasma/blood assays.

Evaluation in Animal Model of Thrombosis

To test the 6 inhibitors in an in vivo setting, we modified a model of venous thrombosis previously used for the positive evaluation of specific inhibitors of fXa, fIXa, and thrombin.^{5,15,19} In the original model, enoxaparin at a plasma concentration of 4 U/mL completely inhibited thrombus growth.⁵ The same dose (200 U/kg plus 2 U/kg per min) attains a lower level of inhibition (50% to 55%) in the

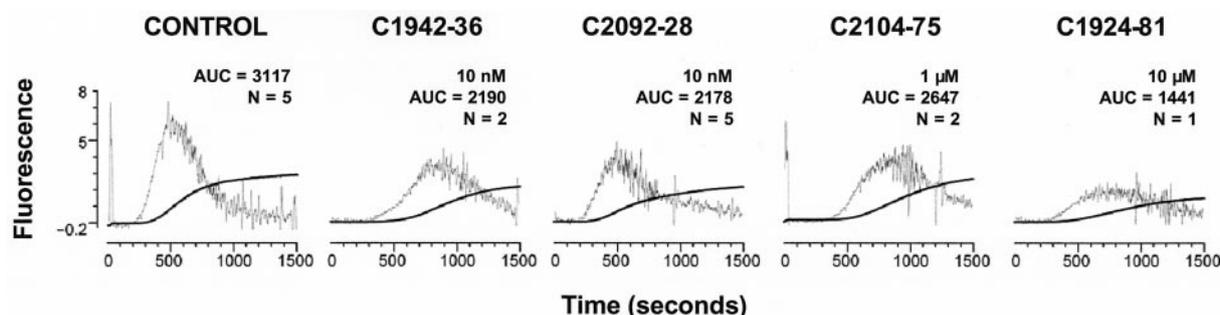


Figure 3. Tissue factor-mediated thrombin generation in human PRP. A representative control ($n=5$) profile for thrombin activity in PRP followed by profiles for each chemical class of inhibitor at 10, 100, or 10 000 nmol/L is shown. The bolded line depicts thrombin activity over time as measured by fluorescent peptide substrate cleavage. The change in relative fluorescence units over time (the derivative) is also shown. The area under the curve was calculated from the derivative of thrombin generation.

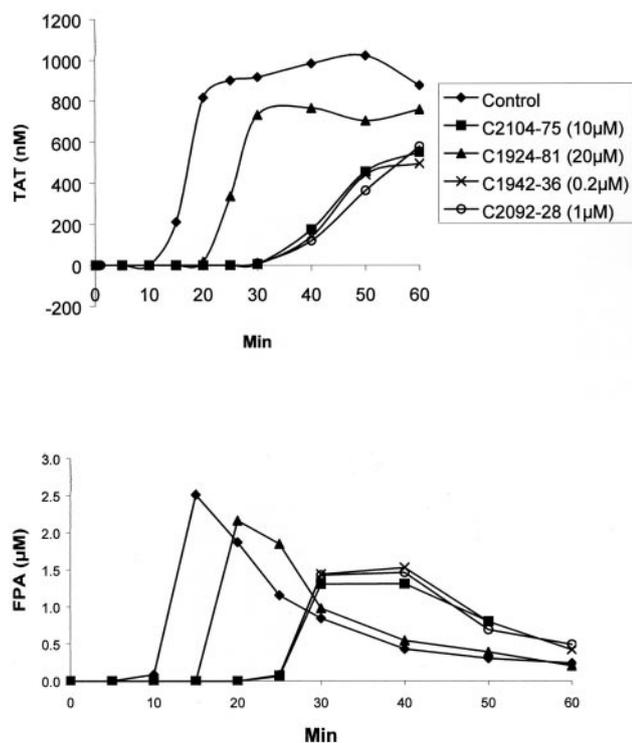


Figure 4. Thrombin generation in whole blood. Two markers of thrombin generation were monitored in gently stirred nonanticoagulated whole blood. Top, Effect of addition of each of 4 fXa inhibitors on the amount of TAT detected by ELISA during the time course of experimentation. Bottom, Amount of FPA detected by LC/MS/MS in the same experimental samples. Each inhibitor was tested 4 times. A representative experiment is shown.

modified protocol. Results of the present study are summarized in Table 1, column 6. Infusion of C2092-28 (total dose, 2 mg/kg) over a 2-hour period produced a 42% inhibition of thrombus accretion at a plasma level of 800 nmol/L and an ex vivo PT change of 1.83-fold. The second P1 benzamidine, C1942-36, was dosed at 0.4 mg/kg and attained inhibition of thrombosis at a plasma level of 200 nmol/L and a corresponding 1.2-fold change over baseline PT. When the aminoisoquinolines were tested at 0.8 mg/kg, C2072-24 had an average plasma level of 210 nmol/L and C1924-81 attained 370 nmol/L, but both had minimal alterations in PT values and no statistically significant inhibition of thrombus weight. Higher doses (2.4 and 1.8 mg/kg) of the aminoisoquinolines

TABLE 2. Inhibition of Prothrombinase Activity in a Reconstituted System

nmol/L	Percent Inhibition of Reconstituted Prothrombinase Complex			
	C1942-36	C2092-38	C2104-75	C1921-81
0	0	0	0	0
10	53.9±2.5	35.2±9.4	16.6±11.0	0
100	91.4±0.8	85.4±2.7	41.0±11.6	19.3±9.6
1000	98.6±0.4	97.5±2.0	87.6±0.7	65.0±4.0

Cleavage of prothrombin 2 by the reconstituted human fXa/fVa complex on phospholipid membranes is used as a measure of prothrombinase activity. Results are shown as average±SEM (n=4).

TABLE 3. Evaluation in a Rabbit Model of Venous Thrombosis

Total Dose of C2104-75, mg/kg	No. of Animals	Inhibition of Thrombosis, %	Plasma Concentration, µmol/L	PT, fold change
0.914	7	9†	0.98	1.07
2.538	3	19†	3.2	1.20*
5.076	3	0†	7	1.25*

Efficacy results for an escalation in administration of C2104-75 are shown. Increased dosing of compound gave a plasma level that reached 7 µmol/L. Despite the change in ex vivo clotting parameters, there was no inhibition of thrombus weight.

*Statistically increased compared with vehicle control group ($P<0.05$).

†Average clot weight of C2104-75-treated groups was not statistically different from vehicle controls as calculated by Student's *t* test.

were still unable to achieve efficacy. At a plasma concentration of 700 nmol/L, C1924-81 extended PT by only 3% over baseline. This agrees with in vitro data that showed 61 µmol/L of this inhibitor was required to produce a 2-fold change in clotting time (data not shown). The 2 neutral inhibitors were investigated in dose escalation studies. C2112-42 was evaluated at 2 doses (0.6 and 1.4 mg/kg), where the higher dose achieved a plasma concentration of 1.3 µmol/L but no antithrombotic activity. As shown in Table 3, the dose escalation of C2104-75 gave increasing plasma concentrations (up to 7 µmol/L) without any significant effect on thrombus weight. Only small increases in PT were noted for the 2 neutral compounds despite high systemic drug concentrations.

Discussion

Generation of thrombin is an integral part of platelet activation and fibrinogen cleavage at the site of vascular injury. Thrombin is the product of a cascade of proteolytic activity that is highly regulated by enzymatic complex formation. FXa, the enzymatic component of the prothrombinase complex, is capable of generating thrombin as an independent enzyme and also as a part of the fVa-bound complex. The complex-bound enzyme is 300 000-fold more efficient in prothrombin cleavage,⁴ so a potential drug candidate has to inhibit both solution-phase fXa and the prothrombinase complex. Ideally it would be advantageous to screen several natural products and synthetic inhibitors against the real target, ie, prothrombinase assembled on activated platelets. The technical complexity of setting up robust screening assays that incorporate human cells has prevented this from being the protocol of choice. Even when the assays are set up using phospholipid membranes, a system that involves a membrane-bound enzymatic complex cleaving a membrane-bound substrate is poorly amenable to generating kinetic parameters in a screening assay. We have measured the inhibitory activity of 4 of the inhibitors studied here in a reconstituted purified prothrombinase assay (Table 2). The results support our assertion that we are measuring prothrombinase activity in our plasma/whole blood assays because the compounds inhibit in a similar rank order.

The in vitro protein binding values in Figure 1 were used to calculate free inhibitor concentrations, and the results were compared among the 3 chemical classes of inhibitors. The

differences in efficacy among the inhibitors tested cannot be explained solely on protein binding, because several measured inhibitor concentrations vastly exceed their putative therapeutic levels.

There are several compromises made in setting up the ex vivo prothrombinase assays investigated in this report. The TF used in both TF-mediated assays (plasma and PRP) is a recombinant purified protein produced in *Escherichia coli*. The recombinant TF is truncated and is artificially lipidated by being combined with synthetic phospholipids. It is the synthetic lipid that most likely accounts for the concentration requirement for added TF being dependent on the presence or absence of platelets. In the absence of platelets (Figure 2), 640 pmol/L TF is used to initiate thrombin generation in reptilase-treated plasma; in the PRP assay (Figure 3), 0.8 pmol/L TF is sufficient for detecting thrombin activity. In addition, in the PRP assay, effects of TF and Pefabloc FG are related to the aggregometer tube being siliconized (Sigma-coted). When comparing the onset of detectable thrombin levels in nonsiliconized versus siliconized tubes in the absence of TF, a greatly increased lag time is noted in a sigma-coted tube. Tubes that have not been siliconized are most likely triggering thrombin generation by TF as well as by the contact pathway initiated on the surface of the tube. In siliconized tubes, the effect of varying concentrations of tissue factor led to dose-dependent changes in thrombin activity, whereas the relationship was variable in noncoated tubes. In addition, for assays carried out in coated tubes, omission of TRAP significantly dampens the rate of thrombin generation. The combination of TRAP and magnetic stirring assures that reproducible platelet aggregation takes place in platelet preparations from different donors. In the thrombin generation assays described here, the clotting of fibrinogen can interfere with detection of signal. In the plasma assay (Figure 2), reptilase treatment is used to remove most of the fibrinogen and thereby prevent clot formation that can interfere with detection of parnitroanilide cleavage. Because clot-bound thrombin is important in propagating the clotting cascade and subsequent platelet activation, this feedback mechanism is completely ignored in the plasma assay. In the PRP assay, the agent Pefabloc FG and stirring are required to prevent clotting, because this leads to irregular fluorescent tracings. When following thrombin generation markers in whole blood, stirring at a consistent rate is required to enable reproducible sampling over the time course of experimentation. Both the PRP and whole-blood assay (Figures 3 and 4, Table 1) are sensitive to rates of stirring, and reproducibility of stirring had to be ensured between different experiments.

In the series of prothrombinase inhibitory assays summarized in Table 1, it is clear that higher concentrations of inhibitor are required for inhibition of thrombin generation than is needed for inhibition of fXa cleavage of tripeptide substrates. This is true for all 3 classes of inhibitors examined in this study. In the plasma assay, the issue of competing with 1.4 $\mu\text{mol/L}$ prothrombin (the zymogen concentration in plasma) may be a contributing factor to the reduced potency across different chemical classes. We have observed that for P1 benzamidines, which are relatively potent in the presence of prothrombin (0.4 $\mu\text{mol/L}$ in the plasma assay and 0.1

$\mu\text{mol/L}$ in the PRP assay), the concentration of inhibitor required for half-maximal inhibition is at least 100-fold greater than the requirements for inhibition of tripeptide substrate cleavage.

Krishnaswamy and Betz²⁰ have reported that the recognition of prothrombin results from an initial interaction at an exosite that is distinct from the protease active site. P-amino benzamidine, an active site inhibitor, behaves as a competitive inhibitor of cleavage of peptide parnitroanilide substrate by fXa. When the same inhibitor is evaluated for its capacity to inhibit prothrombin 2 cleavage by prothrombinase, the kinetics of inhibition follow those of a classical noncompetitive inhibitor. Betz and Krishnaswamy have also shown that inhibition of macromolecular substrate cleavage can be achieved by interfering with exosite binding without blocking the enzyme active site.²¹ Bothrojaracin, a snake venom protein that binds to the proexosite I on prothrombin, can inhibit prothrombin activation by prothrombinase complexes assembled on platelet membranes.²² The inhibitory effect of Bothrojaracin can only be detected when the assays are carried out using the prothrombinase complex and not fXa alone. The results provide additional proof that exosites are involved in productive recognition of prothrombin as substrate. It is possible that the alterations in fXa conformation and activity, which stem from its cofactor binding and incorporation into prothrombinase, have an effect on the inhibition profile of the active site-directed inhibitors being evaluated for drug development. When newly synthesized compounds are screened against purified fXa, the emerging SAR of a particular series is often compared with that of other chemically divergent series. It is important to remember that SAR identified by inhibitory activity against solution phase fXa may be significantly different from the SAR for prothrombinase complex inhibition. This in turn would yield varied correlation with whole blood assays and animal models. Thus, for drug development projects, it may be prudent to consider fXa inhibitory potency as a screening criteria and to use inhibition of prothrombinase as a more predictive tool for identification of candidates for additional evaluation.

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