Articles

Co-crystal Structure and Inhibition of Factor Xa by PD0313052 Identifies Structurally Stabilized Active Site Residues of Factor Xa and Prothrombinase

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ABSTRACT: The enzyme complex prothrombinase plays a pivotal role in fibrin clot development through the production of thrombin, making this enzyme complex an attractive target for therapeutic regulation. This study both functionally and structurally characterizes a potent, highly selective, active site directed inhibitor of human factor Xa and prothrombinase, PD0313052, and identifies structurally conserved residues in factor Xa and prothrombinase. Analyses of the association and dissociation of PD0313052 with human factor Xa identified a reversible, slow-onset mechanism of inhibition and a simple, single-step bimolecular association between factor Xa and PD0313052. This interaction was governed by association (k_{on}) and dissociation (k_{off}) rate constants of $(1.0 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and $(1.9 \pm 0.5) \times 10^{-3}$ s^{-1}, respectively. The inhibition of human factor Xa by PD0313052 displayed significant tight-binding character described by a $K_i^* = 0.29 \pm 0.08$ nM. Similar analyses of the inhibition of human prothrombinase by PD0313052 also identified a slow-onset mechanism with a $K_i^* = 0.17 \pm 0.03$ nM and a k_{on} and k_{off} of $(0.7 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and $(1.7 \pm 0.8) \times 10^{-3}$ s^{-1}, respectively. Crystals of factor Xa and PD0313052 demonstrated hydrogen bonding contacts within the S1−S4 pocket at residues Ser195, Asp189, Gly219, and Gly216, as well as interactions with aromatic residues within the S4 pocket. Overall, these data demonstrate that the inhibition of human factor Xa by PD0313052 occurs via a slow, tight-binding mechanism and indicate that active site residues of human factor Xa, including the catalytic Ser195, are effectively unaltered following assembly into prothrombinase.

At the site of vascular injury, a complex array of procoagulant events occur culminating in the production of the serine protease thrombin, leading directly to fibrin deposition and clot formation (*1*−*6*). The production of thrombin is a tightly regulated process carried out by the enzyme complex prothrombinase, which consists of the serine protease factor Xa, complexed with its cofactor, factor Va, assembled in a calcium dependent manner on the surface of an appropriate phospholipid membrane, such as that presented by activated platelets in vivo (*1*, *3*, *6*).

Although factor Xa is capable of producing thrombin from the zymogen prothrombin in solution, assembly into prothrombinase accelerates the efficiency of the reaction by five orders of magnitude representing the physiologically relevant reaction rate (*7*, *8*). This dramatic increase in catalytic activity is a cumulative result of interactions between all members of prothrombinase. However, factor Xa combined with high concentrations of factor Va alone yields rates of thrombin generation on the order of the entire complex, thus, it is believed that factor Va contributes significantly to modulating factor Xa catalytic activity (*9*). Recent evidence suggests that the primary function of factor Va is to perturb exosites on factor Xa that serve to bind the substrate and direct the scissile bonds of prothrombin to the active site contained in factor Xa (*10*, *11*).

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An increasing body of evidence suggests that the active site of factor Xa may be relatively unaltered by assembly into the prothrombinase complex. Whereas studies monitoring changes in fluorescence of the irreversible inhibitor DEGR-ck to bovine factor Xa identified significant alterations in fluorescence intensity following binding of the inhibitor to factor Xa (*12*−*15*), the presence of factor Va was shown to have little effect on the alkylation of the inhibitor and hence on the function of the catalytic triad (*15*). Therefore, significant structural alterations in the bovine factor Xa active site were not observed as a result of complex assembly. Interestingly, no increase in fluorescence quantum yield is observed from DEGR-ck using human factor Xa, suggesting that the chemical environment is distinctly different in the active site of the two species. To date, the best probe for the analysis of the human factor Xa active site has been Oregon $Green_{488}$ tethered to an active site binding peptide. Although this probe displays a shift in anisotropy following assembly of prothrombinase (*11*), no specific structural information is yet available to discern the identity of regions surrounding the active site that lead to the change in quantum environment. Therefore, the structure of the active site of human factor Xa as it assembles into prothrombinase has yet to be fully explored.

The current investigation examines the mechanisms of binding of a small molecule inhibitor of human factor Xa, PD0313052, which was discovered by medicinal chemistry

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optimization of positive hits from high-throughput screening. PD0313052 was developed as a parenteral factor Xa inhibitor and is based on an aryl-amidine group designed to fit deep in the S1 pocket. Amidine mimics are a hallmark of factor Xa inhibitors such that they imitate the substrate arginine at the P1 site. This study examines the inhibition kinetics of PD0313052 as it interacts with factor Xa and prothrombinase and further identifies interactions with the factor Xa S1−S4 pocket as present in the co-crystal structure of factor Xa/PD0313052 that suggest structural identity in factor Xa and prothrombinase.

EXPERIMENTAL PROCEDURES

Reagents. L-α-Phosphatidylserine [bovine brain] (PS) and L-α-phosphatidylcholine [egg yolk] (PC) were purchased from Sigma (St. Louis, MO). Crystallized bovine serum albumin was purchased from Serological Proteins Inc. (Kankakee, IL). Dansylarginine *N*-(3-ethyl-1,5-pentanediyl)-amide (DAPA[1]) and coagulation factors Xa, V, prothrombin, and α-thrombin were purchased from Haematologic Technologies Inc (Essex Junction, VT). Factor Xa for crystallographic analyses was purchased from Enzyme Research Lab (South Bend, IN). All coagulation proteins were of human origin. The direct thrombin inhibitor hirudin was purchased from Genentech (South San Francisco, CA). The active site directed factor Xa inhibitor PD0313052 was synthesized by Pfizer Global Research and Development (Ann Arbor, MI). S-2222 was purchased from Chromogenix (Milano, Italy). The fluorescent substrate FS-2765 was purchased from California Peptide Research (Napa, CA). Synthetic phospholipid vesicles composed of 75% (% wt/wt) phosphatidylcholine (PC) and 25% (% wt/wt) phosphatidylserine (PS) were prepared as described by Barenholz et al. (*16*). Concentration was determined by phosphorus assay as described (*17*). Molecular weights and extinction coefficients ($E^{1\%}_{280\ nm}$) of the proteins used were taken as follows: prothrombin 72 000 Da 14.2 (*18*), thrombin 37 000 Da 1.74 (*19*), factor V 330 000 Da, 9.6 (*20*), and factor Xa 46 000 Da, 11.6 (*18*). The fluorescent substrate (Z-Gly-Gly-Arg-AMC) was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). Microtiter plates used were half-area 96-well, flat bottom, nonbinding surface, black polystyrene plates purchased from Corning, Inc. (Corning, NY).

Progress of the Inhibition of Factor Xa and Prothrombinase by PD0313052. Progress curves were obtained by monitoring the turnover of the small substrate FS-2765 by 50 pM factor Xa in assay buffer (20 mM HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, 0.1% BSA, pH 7.4) at ambient temperature. Solutions of FS-2765 with varying concentrations of PD0313052 (0−3 nM final) were added to factor Xa, and the progress of substrate turnover was monitored with $\lambda_{ex} = 390$ nm and $\lambda_{em} = 460$ nm until equilibrium was well established (approximately 45 min). Progress curves of preformed prothrombinase were carried out using 50 pM factor Xa, 20 nM factor Va, and 20 μM PCPS vesicles (final concentrations) in assay buffer. The concentrations of factor Va and PCPS vesicles were such that greater than 99% of the factor Xa was bound in prothrombinase. Solutions of varying concentrations of PD0313052 were combined with FS-2765 such that the final assay concentrations were 200 μM FS-2765 and 0−3 nM PD0313052.

Determination of the Overall Dissociation Constants for Inhibition of Human Factor Xa and Prothrombinase by PD0313052. The overall dissociation constants of inhibition of factor Xa and prothrombinase by PD0313052 were determined by incubating increasing concentrations of the inhibitor in 75 μL reaction mixtures containing either (a) 0.25 nM factor Xa or (b) 0.4 nM factor Xa plus 20 μM PCPS vesicles and 5 nM plasma-derived factor Va in assay buffer. Residual enzyme activity was assayed following a 60 min incubation at room temperature. The reactions were started by the addition of 25 μL of FS-2765 (200 μM final), prepared in the appropriate buffer. Measurements of turnover of FS-2765 were obtained at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 460$ nm on a Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA). To further evaluate the inhibition of prothrombinase by PD0313052, a discontinuous assay system was employed to monitor the conversion of the natural macromolecular substrate prothrombin to thrombin in the above preincubated enzyme−inhibitor mixture. Measurements of prothrombin turnover were obtained in reaction mixtures prepared as described above followed by the addition of 25 μL of prothrombin and DAPA to final concentrations of 1.39 μM and 3 μM, respectively. At regular intervals a small aliquot (10 μL) was removed and diluted into 140 μL of quench buffer (20 mM HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, 50 mM EDTA, 0.1% PEG-8000). To this was added 50 μL of S2238 (Chromogenix, Milano, Italy) for a final concentration of 0.2 mM, and the absorbance was monitored using a Spectramax 250 plate reader (Molecular Devices, Sunnyvale, CA). The amount of thrombin generated at each time point was determined by comparison to a thrombin standard curve.

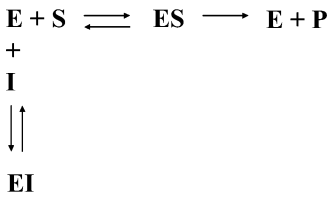
Reversibility of the Inhibition of Human Factor Xa by PD0313052. To determine if the interaction of human factor Xa and PD0313052 was reversible, dilution experiments were performed. Factor Xa (5 nM) was incubated with various concentrations of PD0313052 (0−200 nM) for 60 min at ambient temperature to allow for a binding equilibrium to develop. The inhibited complex was then diluted 100-fold in assay buffer containing 200 μM (final) FS-2765, and substrate turnover was monitored as described above. Data were analyzed by nonlinear least squares regression analysis using eq 1 describing slow-binding inhibition with the appropriate substitutions (*21*).

Measurements of Thrombin Generation in Human Plasma. In a nonbinding, half-area 96-well assay plate, 17.5 μL of buffer A (20 mM HEPES, 150 mM NaCl, 0.1% BSA, pH 7.4) was added to 2.5 μL of respective inhibitor compound and 80 μL of pooled normal human plasma, and the mixture was incubated for 15 min at 37 °C. The fluorescent substrate, Z-Gly-Gly-Arg-AMC, was reconstituted to 5 mM in buffer A and mixed with Innovin (1:100 dilution in buffer A plus 750 mM $CaCl_2$) at a 2:1 ratio, respectively. Activation of the plasma began with addition of 30 μL of the substrate and activator solution, and the resulting increase in fluorescence was measured at $\lambda_{ex} = 390$ nm, $\lambda_{em} = 460$ nm, with a cutoff at 455 nm for 60 min at 37 °C.

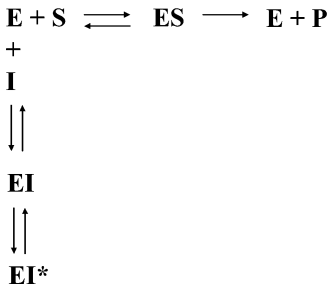
Data Analysis. Multiple mechanisms of slow-binding inhibition have been proposed (*21*−*24*), which differ in the

[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; PCPS, phosphatidylcholine phosphatidylserine vesicles (wt % 75:25); DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; FXa, human coagulation factor Xa; FVa, human coagulation factor Va.

Scheme 1



Scheme 2



methods of association between the enzyme and inhibitor. Scheme 1 involves only the observation of a direct single-step association of enzyme and inhibitor in which the slow onset of equilibrium observed in progress curves is primarily a result of a slow dissociation rate (k_{off}) relative to the rate of association (k_{on}) governing the equilibrium between the enzyme and the substrate. Scheme 2 involves an initial rapidly developing equilibrium formed between the enzyme and the inhibitor. However, this mechanism includes a second phase of slowly established equilibrium representing a more tightly bound enzyme−inhibitor complex (EI*).

$$V_o = \frac{k_{cat}E_TS_o}{K_m + S_o}; \qquad k_{obs} = k_{off} + \frac{k_{on}I_T}{1 + S_o/K_m} \tag{1}$$

$$V_o = \frac{k_{cat}E_TS_o}{K_m(1 + I_T/K_i) + S_o},$$

$$k_{obs} = k_{off} + \frac{k_{on}I_T}{K_i(1 + S_o/K_m) + I_T} \tag{2}$$

where k_{obs} represents the observed first-order rate of the onset of inhibition following addition of inhibitor and substrate, k_{on} and k_{off} represent the rate constants describing the second-order association and first-order dissociation between the enzyme and the inhibitor respectively, S_o is the initial substrate concentration, I_T is the total inhibitor concentration, and V_o represents the observed initial velocity of product formation. The two schemes can be readily distinguished by the manner in which V_o and k_{obs} vary with inhibitor concentration. Scheme 1 yields a single-step association between inhibitor and enzyme such that the V_o is independent of [I], and k_{obs} varies linearly with [I]. Scheme 2 describes a multistep association in which both V_o and k_{obs} vary hyperbolically with [I]. Both schemes can be analyzed by a common equation (eq 3) describing the progress of product formation, provided that pseudo-first-order conditions prevail in that unappreciable substrate depletion occurs and the concentration of E is much less than the concentration of I (*22*, *25*):

$$P = V_s t + (V_o - V_s)(1 - e^{-k_{obs}t})/k_{obs} \tag{3}$$

where V_o represents the initial velocity, V_s is the steady-state velocity following the establishment of an equilibrium between the enzyme and inhibitor, and k_{obs} represents the observed overall first-order rate constant describing the rate of onset of inhibition resulting from association between the enzyme and the inhibitor.

Progress curves monitoring the association of the enzyme−inhibitor complex were analyzed by eq 3, fitting for V_o, V_s, and k_{obs} using nonlinear regression analysis with the software package Prism 2.01 (Graphpad Software Inc., San Diego, CA). The values obtained for k_{obs} were subsequently plotted against the varying concentrations of PD0313052. A linear relationship allowed for the use of eq 1 for linear fitting to obtain the parameter k_{on} and an estimate of k_{off}. As with most slow, tight-binding inhibitors Scheme 2 is used to fully describe the system. However, the relative rates of dissociation between the E and I, the EI complex, and the EI* complex were such that the inhibition of human factor Xa and prothrombinase by PD0313052 could be described by the more simple system represented by Scheme 1.

Analyses of dissociation experiments were performed as previously described (*21*), using global curve fitting to eq 3 substituting the values described in eq 1 and taking into account the dilution factor of 100 necessary to carry out the experiments. Dissociation experiments of the inhibition of human prothrombinase by PD0313052 were not carried out to determine a more accurate estimate for the k_{off} as the large dilution necessary to observe dissociation also alters the equilibrium of the components of the enzyme complex. Thus, the k_{off} value obtained from analyses of the association experiments is reported.

Determination of the Overall Dissociation Constant, K_i.* Initial velocity measurements were obtained at a fixed concentration of enzyme following incubation with the inhibitor PD0313052 according to eqs 4 and 5 (*26*) to obtain fitted values for K_i*, υ_o, and υ_∞.

$$E_i = \frac{nI + E + K_i^* - \sqrt{(nI + E + K_i^*)^2 - 4nIE}}{2} \tag{4}$$

$$\upsilon_{obs} = \upsilon_\infty E + \upsilon_o E\left(1 - \frac{E_i}{E}\right) \tag{5}$$

The total concentrations of the enzyme, prothrombinase, and the inhibitor, PD0313052, are described by E and I, respectively; E_i is the concentration of the inhibited enzyme; K_i* is the overall dissociation constant for the binding interaction between the slow, tight-binding inhibitor and the enzyme; and n is the moles of I bound per mole of E at saturation. The stoichiometry value, n, was held constant at a value of 1.

Additionally, K_i* was calculated from the equation

$$K_i^* = \frac{k_{off}}{k_{on,app}} = \frac{k_{off}}{k_{on}/(1 + S_o/K_m)} \tag{6}$$

where the K_m of substrate turnover was experimentally determined as 178 μM. The independently measured values of k_{on} and k_{off} provided estimates of K_i*.

Crystallization of PD0313052/Factor Xa, Structure Solution, and Refinement. Co-crystals of human des Gla Factor Xaβ protein were produced by hanging drop vapor diffusion

Table 1: Crystallographic Data of the FXa−PD0313052 Binary Complex

data collection	2BJ5 (PDB code)
space group	$P2_12_12_1$
unit cell dimensions (Å)	$a = 55.815$
	$b = 71.855$
	$c = 77.813$
resolution range (Å)	50.0−2.0
observations	98053
unique observations	20674
completeness (outermost shell)	94.1 (71.1)
R_{sym} (%) (outermost shell)	6.1 (29.0)
$I/\sigma(I)$ (outermost shell)	27.65 (2.47)
refinement	
resolution (Å)	50.0−2.0
$\|F\|/\sigma(\|F\|)$	>0
R_w/R_f	20.8/25.2
reflections (working/test)	17777/1948
protein atoms	2229
solvent molecules	192
rmsd bond lengths[a] (Å)	0.005
rmsd angles[a] (deg)	1.25
rmsd *B* values (Å) (mc/sc)[b]	1.6/2.1
⟨B⟩ protein (Å^2)	38.4
⟨B⟩ solvent (Å^2)	46.8

[a] Root-mean-squared deviation (rmsd) from ideal bond lengths and angles and rmsd in *B*-factors of bonded atoms. [b] mc, main chain; sc, side chain.

using a reservoir solution composed of 25% PEG 600, 0.3 M NaCl and 0.1 M MES buffer (pH = 5.9). Des Gla Factor Xaβ protein (8 mg/mL) complexed with 0.1 mM PD0313052 was mixed with an equivalent volume of reservoir solution and equilibrated over 250 μL of reservoir solution. Single, rod shaped crystals (60 × 60 × 240 μm) grew in 6 weeks as a result of microseeding 2 days after setup. Crystals were quickly dipped in oil (70% Paratone-N (Hampton Research, HR2-643) and 30% light white mineral oil (Sigma, M-3516) and flash-cooled in liquid nitrogen. The crystal diffracted X-rays to 2.0 Å resolution under cryogenic conditions at the IMCA-CAT 17-ID beamline at the Advanced Photon Source (Argonne National Laboratories, Argonne, IL). Crystals are of the orthorhombic space group $P2_12_12_1$ [$a = 55.82$ Å, $b = 71.86$ Å, and $c = 77.81$ Å with one molecule in the asymmetric unit (Matthews coefficient of 2.09 Å^3/Da and 41.15% solvent content)] (Table 1).

RESULTS

Mechanism of Factor Xa Inhibition by PD0313052. To determine the mechanism of inhibition of human factor Xa by PD0313052, progress curves resulting from measurements of the turnover of the small fluorescent peptidyl-substrate FS-2765 were obtained. Factor Xa was added to reaction mixtures containing the indicated concentrations of PD0313052 in substrate. Measurements of product formation over time yielded rapid initial rates, followed by a transient phase to a second, slower rate of hydrolysis representing a slow-onset equilibrium between factor Xa and PD0313052 (Figure 1A). These progress curves were fitted to eq 3 to generate fitted values for V_o, V_s, and k_{obs}. The initial rate, V_o, was wholly independent of PD313052 concentration, while the observed second-order rate constant, k_{obs}, increased linearly as the concentration of inhibitor was increased, suggesting that the second association step of slow, tight-binding inhibitors is kinetically hidden and allowing for the use of equations

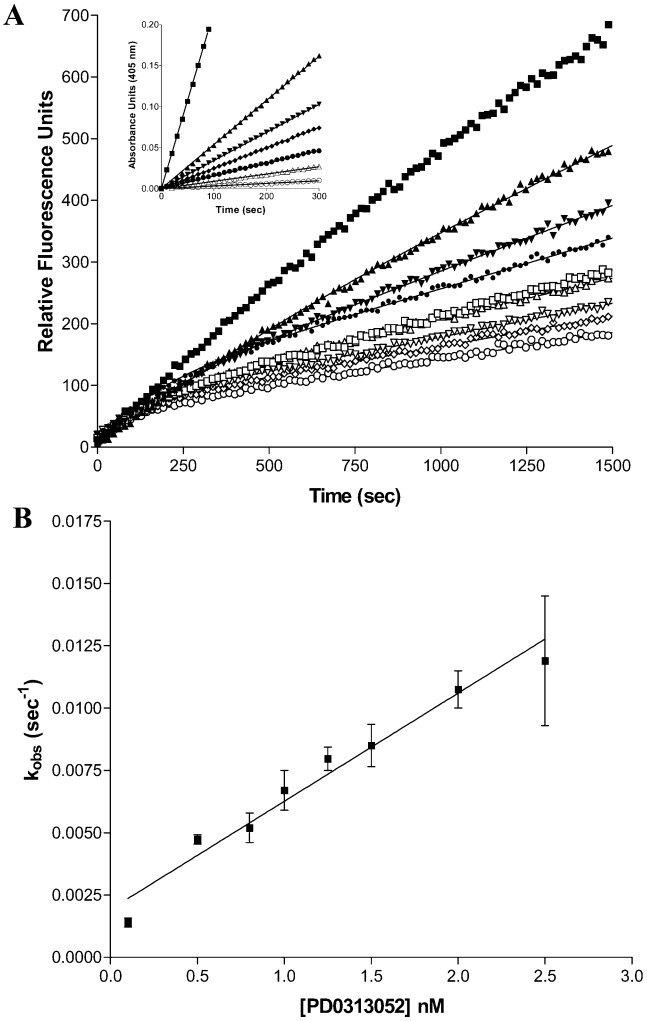


FIGURE 1: (A) Inhibition of human factor Xa by PD0313052. Progress curve analyses were started by the addition of human factor Xa (50 pM final) to mixtures of FS-2765 (200 μM final) and various final concentrations of PD0313052: ■ 0 nM, ▲ 0.1 nM, ▼ 0.5 nM, ● 0.8 nM, □ 1.25 nM, △ 1.5 nM, ▽ 2 nM, ◇ 2.5 nM, and ○ 3 nM in assay buffer. Slow-onset inhibition is marked by the decrease in the rate of product formation over time. (Inset) Progress curves of the inhibition of human factor Xa by the simple competitive inhibitor DX-9065a. Final concentrations of DX-9065a were ■ 0 nM, ▲ 100 nM, ▼ 200 nM, ◆ 300 nM, ● 500 nM, △ 1 μM, and ○ 2 μM. The linear rate of product formation indicates a rapidly developed equilibrium between DX-9065a and factor Xa. (B) Dependence of k_{obs} on the concentration of PD0313052. Values for k_{obs} were obtained by fitting the data presented in panel A to eq 3. Values are representative of nine independent experiments. The data were fit to eq 1 to obtain the values $k_{on} = (1.0 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and $k_{off} = (1.9 \pm 0.5) \times 10^{-3}$ s^{-1}.

describing a more simple, single-step association process (Figure 1B).

Equation 1 was used to determine values for the relative association and dissociation rate constants k_{on} and k_{off} of (1.0 ± 0.1) × 10^7 M^{-1} s^{-1} and (1.9 ± 0.5) × 10^{-3} s^{-1}, respectively (Figure 1B). These values indicated a fast, nearly diffusion limited, association between factor Xa and PD313052, with the relatively slow dissociation producing the overall slow onset of equilibrium. Comparative experiments carried out using the competitive factor Xa inhibitor DX-9065a demonstrated the expected fast-binding character (Figure 1, inset) (*27*, *28*).

PD0313052 Associated with Human Factor Xa in a Reversible Manner. To decipher whether PD0313052 inter-

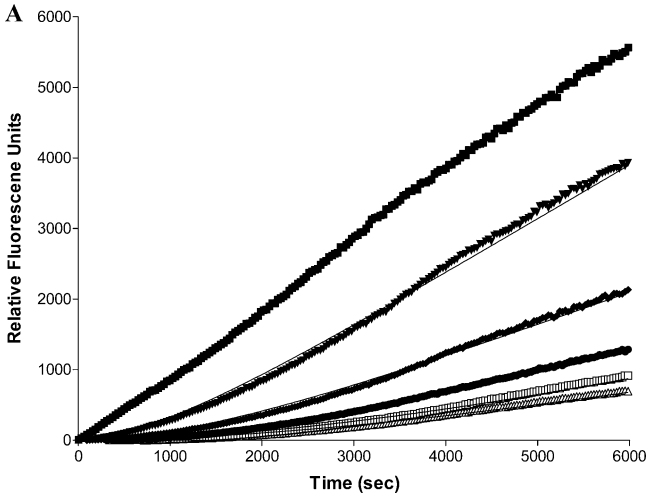


FIGURE 2: The inhibition of human factor Xa by PD0313052 is reversible. Human factor Xa (5 nM) was incubated for 60 min at ambient temperature with increasing concentrations of PD0313052 (■ 0 nM, ▼ 0.1nM, ◆ 0.5 nM, ● 1 nM, □ 1.5 nM, △ 2 nM, and final concentrations following dilution). The equilibrated reaction mixtures were then diluted 100-fold into FS-2765 (300 μM final) to start the reaction, and progress was monitored over the course of time. Global analyses using eq 3 with the appropriate substitutions (*21*) yielded $k_{off} = (1.0 \pm 0.3) \times 10^{-3}$ s^{-1}, in excellent agreement with values obtained in experiments monitoring the onset of inhibition of factor Xa by PD0313052 (Figure 1A,B). Data are representative of three independent experiments.

acted with factor Xa reversibly, dissociation experiments were performed as described in Experimental Procedures. Progress curve analyses in which factor Xa was preincubated with PD0313052 for 60 min, followed by a 100-fold dilution into FS-2765, demonstrated increased rates of substrate cleavage recovered over time indicating that the interaction between PD0313052 and factor Xa was reversible (Figure 2). Fitting the dissociation experiments by global analyses to eq 3 with the appropriate substitutions (*21*) yielded a k_{off} of $(1.0 \pm 0.3) \times 10^{-3}$ s^{-1}, in agreement with the estimated value of $(1.9 \pm 0.5) \times 10^{-3}$ s^{-1} obtained in analyses of the association experiments.

PD0313052 Inhibits Human Prothrombinase Indistinguishably from Factor Xa. The catalytic efficiency of prothrombin turnover by factor Xa increases ~300000-fold following its incorporation into prothrombinase (*7*, *8*). Thus, significant structural alterations may occur in factor Xa due to interactions with factor Va and phospholipid that may modify the interactions of PD0313052 with the protease active site. Thus, the association of PD0313052 with prothrombinase was analyzed. Similar to solution phase factor Xa, the inhibition of prothrombinase by PD0313052 was described by a slow-onset mechanism of inhibition (Figure 3A) with k_{obs} linearly dependent on the concentration of PD0313052, indicating a single-step mechanism of association between prothrombinase and PD0313052 (Figure 3B). Analyses of the dependence of k_{obs} on the concentration of PD0313052 by eq 1 yielded rates for k_{on} and k_{off} of $(0.7 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and $(1.7 \pm 0.8) \times 10^{-3}$ s^{-1}, respectively. The high error value for k_{off} demonstrates the difficulty of inferring this value from experiments analyzing association of the enzyme inhibitor complex. Nonetheless, these values are virtually identical to that of factor Xa in solution and suggest that the assembly of factor Xa into prothrombinase did not appreciably alter the S1−S4 pocket of the protease.

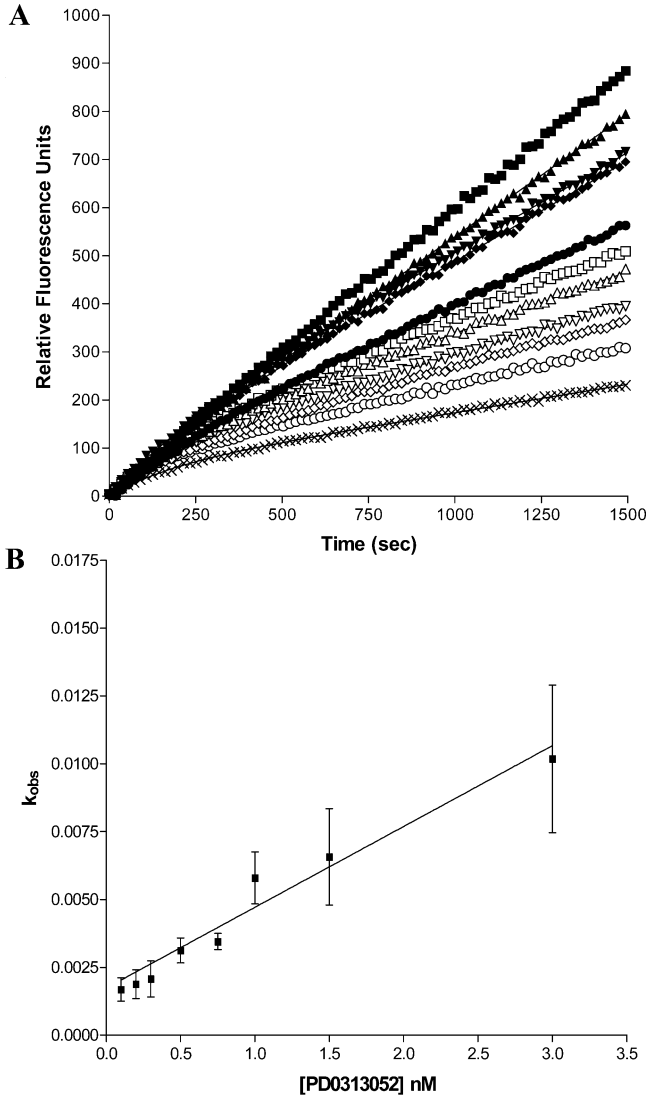


FIGURE 3: (A) Inhibition of human prothrombinase by PD0313052. Progress curve analyses were started by the addition of human prothrombinase (50 pM factor Xa, 20 nM factor Va, and 20 μM PCPS vesicles (final)) to solutions containing FS-2765 (200 μM final) and varying final concentrations of PD031302: ■ 0 nM, ▲ 0.1 nM, ▼ 0.2 nM, ◆ 0.3 nM, ● 0.5 nM, □ 0.75 nM, △ 1.0 nM, ▽ 1.25 nM, ◇ 1.5 nM, ○ 2 nM, and × 3 nM in assay buffer. The concentrations of factor Va and PCPS vesicles were such that greater than 99% of the factor Xa was bound in prothrombinase. Slow-onset inhibition is marked by the decrease in product formation rate over time. (B) Dependence of k_{obs} on the concentration of PD0313052 for the inhibition of prothrombinase. Values for k_{obs} were obtained by fitting the data presented in panel A to eq 3. Values are representative of five independent experiments. The data were fit to eq 1 to obtain the values k_{on} and k_{off} of (0.7 ± 0.1) M^{-1} s^{-1} and $(1.7 \pm 0.8) \times 10^{-3}$ s^{-1}, respectively.

Additional experiments were carried out to decipher the overall dissociation constants, K_i*, for the binding of PD0313052 to both factor Xa and prothrombinase. Steady-state binding measurements of small peptidyl substrate turnover were performed at increasing concentrations of PD0313052 following a 60 min incubation. The inhibition of both factor Xa and prothrombinase by PD0313052 exhibited significant tight-binding character with no observable difference in the overall dissociation constant for the inhibitor binding to either protease, resulting in $K_i^* = 0.29 \pm 0.08$ nM and 0.17 ± 0.03 nM for factor Xa and prothrombinase, respectively (Figure 4A,B). Additional

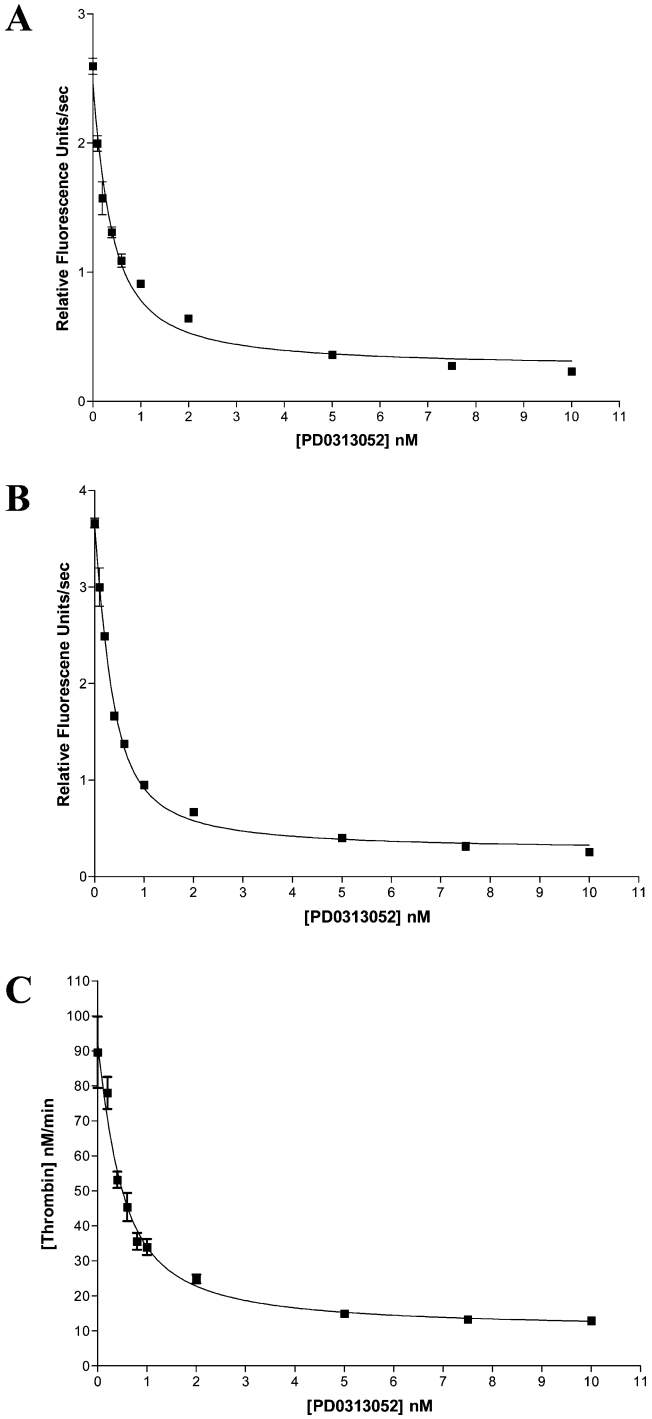


FIGURE 4: Determination of the overall dissociation constant, K_i*, for the inhibition of human factor Xa and prothrombinase by PD0313052. Reaction mixtures containing the indicated concentrations of PD0313052 and (A) factor Xa (0.25 nM), (B) prothrombinase (0.4 nM Xa, 5 nM plasma-derived factor Va, and 20 μM PCPS vesicles) in assay buffer or (C) prothrombinase utilizing prothrombin as substrate were incubated for 60 min at ambient temperature. Reactions were initiated with (A and B) FS-2765 (200 μM final) or (C) a mixture of prothrombin and DAPA (1.39 μM and 3 μM respectively final) in assay buffer. Steady-state velocity measurements for each reaction type were obtained as described in Experimental Procedures, and the data were analyzed by nonlinear least squares regression analysis using eq 4. The representative value of $K_i^* = 0.29 \pm 0.08$ nM was obtained for the inhibition of factor Xa. PD0313052 also displayed a K_i* of 0.17 ± 0.03 nM and 0.25 ± 0.05 nM for the inhibition of prothrombinase using the substrates FS-2765 and prothrombin, respectively. Data display the results of three independent experiments each.

measurements of the ability of PD0313052 to inhibit the turnover of the physiological substrate prothrombin indicated that the presence of prothrombin does not appreciably alter the binding interactions of PD0313052 with prothrombinase, producing $K_i^* = 0.25 \pm 0.05$ nM (Figure 4C). Comparative experiments to determine the dissociation constant for the factor Xa/DX-9065a interaction were performed, producing $K_i = 35.7 \pm 2.8$ nM (data not shown), in excellent agreement with other investigators (*27*). The indistinguishable values for K_i* and k_{on} observed with the inhibition of factor Xa and prothrombinase by PD0313052 (Table 2) implies similar mechanisms of association and dissociation with the proteases and suggests structural resemblance of the two active sites.

PD0313052 Inhibits Human Prothrombinase Efficiently in Human Plasma. To determine the effects of PD0313052 on prothrombinase catalyzed thrombin generation in plasma, experiments were performed monitoring continuous thrombin generation over time. Due to the nature of this assay system, no inhibition of factor Xa is observed prior to the addition of $CaCl_2$ and tissue factor to start the reaction. The presence of PD0313052 showed a considerable increase in the time required to generate 10 nM thrombin, the amount generally thought to be necessary for in vitro clot formation (Figure 5A). Additionally, increasing concentrations of PD0313052 led to a decrease in both the peak level of functional thrombin available and the total thrombin generated over the course of the reaction. Analyses of the decrease of total thrombin generated as a function of PD0313052 concentration yielded an $IC_{50} = 384 \pm 42$ nM in human plasma (Figure 5B). This value is 1300-fold greater than the K_i* generated in the purified system and may more accurately reflect the concentration necessary for inhibition of prothrombinase in vivo.

Crystal Structure of the Factor Xa/PD0313052 Complex. As the kinetic parameters describing the association of PD0313052 with factor Xa and prothrombinase indicated virtually identical active site structures, we sought to determine the residues within factor Xa that are responsible for binding PD0313052. Therefore, we solved the co-crystal structure of PD0313502 with factor Xa and have deposited the coordinates in the Protein Data Bank (ID code: 2BJ5). A schematic of the noncovalent interactions mediating the binding of PD0313052 to factor Xa is presented in Figure 6A. Factor Xa proteolyzes prothrombin at the amide bond C-terminal to Arg271 and Arg319. The structure of PD0313052 in the active site of factor Xa demonstrated significant interactions that may be well represented by interactions with the P1−P4 residues in prothrombin (Figure 6B). The S1 site of factor Xa contains an aspartic acid at position 189, and the negatively charged side chain of this residue is expected to make two strong hydrogen bonds with the positively charged guanidine end of the arginine in prothrombin. PD0313052 appeared to mimic the arginine of the substrate with a positively charged amidine fragment that paired with the acid side chain of Asp189 of factor Xa, forming a bridge making hydrogen bonds where the amidine donates four strong hydrogen bonds respectively to Gly219O (3.3 Å), Asp189OD1 (2.6 Å), Asp189OD2 (2.80 Å), and a water molecule (2.9 Å). The binding affinity of PD0313052 was significantly strengthened by this salt bridge since ligands that lack this amidine fragment bind to factor Xa with ~100-fold less affinity (data not shown). The phenyl

Table 2: Kinetic Constants Derived from Experiments Monitoring the Association and Dissociation of PD0313052 and DX-9065a with Human Factor Xa and Prothrombinase

enzyme	substrate	inhibitor	mechanism[a]	K_i* (nM)	k_{on} ($\times 10^7$ M^{-1} s^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})
Xa	FS-2765	DX-9065a	competitive	35.7 ± 2.84		
Xa	FS-2765	PD313052	slow	0.29 ± 0.08	1.0 ± 0.1	1.0 ± 0.3[b]
prothrombinase	FS-2765	PD313052	slow	0.17 ± 0.03	0.7 ± 0.1	1.7 ± 0.8
prothrombinase	prothrombin	PD313052	slow	0.25 ± 0.05		

[a] Slow: slow, tight-binding inhibition. [b] Value reported from dissociation experiments.

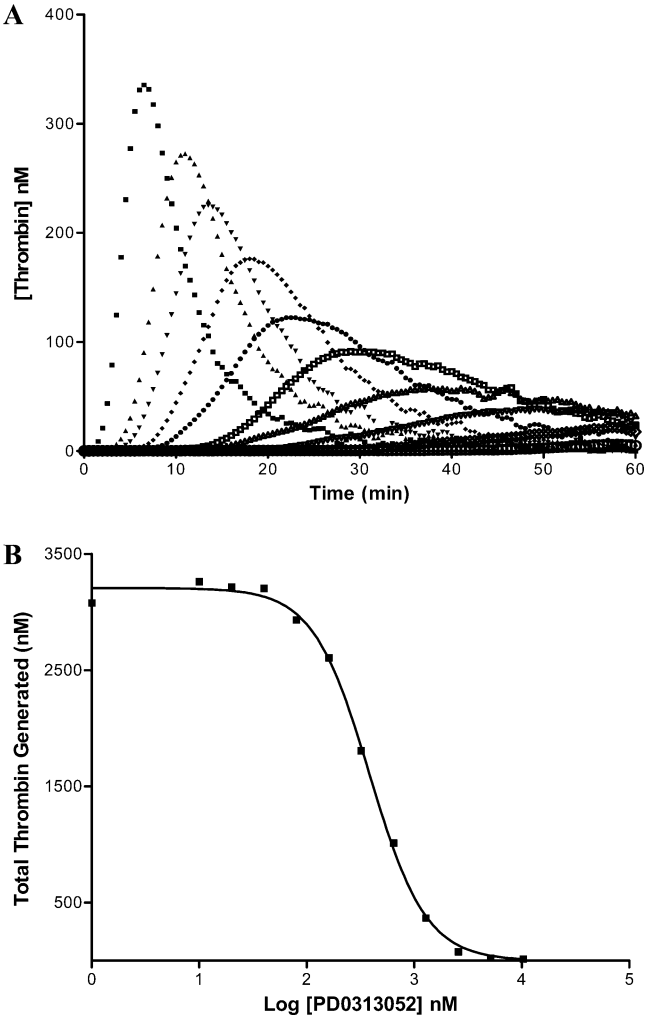


FIGURE 5: The inhibition of prothrombinase by PD0313052 in normal human pooled plasma. (A) The clotting of pooled normal human plasma was activated by the addition of the extrinsic activator Innovin (diluted 100-fold) mixed 1:2 with the fluorogenic thrombin substrate Gly-Gly-Arg-AMC and various concentrations of PD0313052 (■ 0 nM, ▲ 10 nM, ▼ 20 nM, ◆ 40 nM, ● 80 nM, □ 160 nM, △ 320 nM, ▽ 640 nM, ◇ 1.28 μM, ○ 2.56 μM, × 5.12 μM, and + 10.2 μM). The progress of thrombin generation was monitored over 60 min. PD0313052 showed a dose dependent inhibition of human thrombin generation. (B) Dose response of the inhibition of total thrombin generated on the concentration of PD0313052. Data were analyzed by nonlinear least squares regression analyses yielding an $IC_{50} = 384 \pm 42$ nM. Data are representative of three independent experiments.

group of PD0313052 occupied the volume which is accessed by the side chain methylene atoms of the scissile arginines in prothrombin. The phenolic hydroxyl group on the ring para to the amidine accepts a hydrogen bond from the hydroxyl group on the catalytically important Ser195 (inter heavy atom distance 2.6 Å), and this hydrogen bond contributes to the binding affinity. Additional interactions between factor Xa and PD0313052 were found in the heterocyclic diazaquinolinone ring of the inhibitor that bound in the S1β pocket of the enzyme. The carbonyl oxygen accepts a hydrogen bond (inter heavy atom distance 3.0 Å) from the Gly216 amide in factor Xa. Studies of small molecule serine protease inhibitors have suggested that this interaction is important for ligand complexing to serine proteases (*29*, *30*) and is present in most serine protease−peptide complexes utilizing the P3 residue of the substrate. The alkyl chain of PD0313052 made a van der Waals contact with the surface of the enzyme and positioned most of the dimethyl piperidine in the S4 pocket of factor Xa. Three aromatic rings, those of Tyr99, Phe174, and Trp215, surrounded this site. The presence of these three aromatic rings and their π-electrons makes this an attractive region for cationic moieties to bind. In the tick anticoagulant peptide bound structure, the side chain guanidine of Arg503 binds in this pocket (*31*), highlighting the preference for cationic groups to interact with this region in factor Xa. The dimethyl piperidine of PD0313052, which is most likely protonated and therefore cationic, should bind with high affinity in this site. The sum total of the interactions of PD0313052 and factor Xa, where the S1, S3, and S4 sites were filled with high affinity fragments that largely mimic substrate-like features, produced this subnanomolar ligand.

DISCUSSION

There is great interest in the design of direct inhibitors of factor Xa for the therapeutic regulation of thrombin production and thrombotic events (*32*). The great majority of these inhibitors are targeted to the active site of factor Xa for the purpose of competitively inhibiting protease function. However, structural alterations have been proposed to occur in factor Xa following its incorporation into prothrombinase, possibly reducing the validity of drug design directed toward the structure of the solution phase protease. This study demonstrates, using a direct active site binding inhibitor of factor Xa, that the active site of the solution protease is kinetically indistinguishable from the actual therapeutic target, prothrombinase.

Factor Xa acts as a neutral protease between the two extremes of the highly specific protease, thrombin, and the highly prolific protease, trypsin. Factor Xa has the ability to recognize and cleave factor X (*33*), as well as prothrombin (*7*), factor V (*34*), factor VII (*35*), factor VIII (*36*), and factor IX (*37*), indicating that there is a relatively low degree of macromolecular substrate specificity in the absence of the other prothrombinase components. Additionally, small substrate probes directed toward the active site of factor Xa have demonstrated little sequence specificity with only a small range of catalytic activities toward peptides with a wide range of amino acid substitutions (*38*). The relative promiscuity

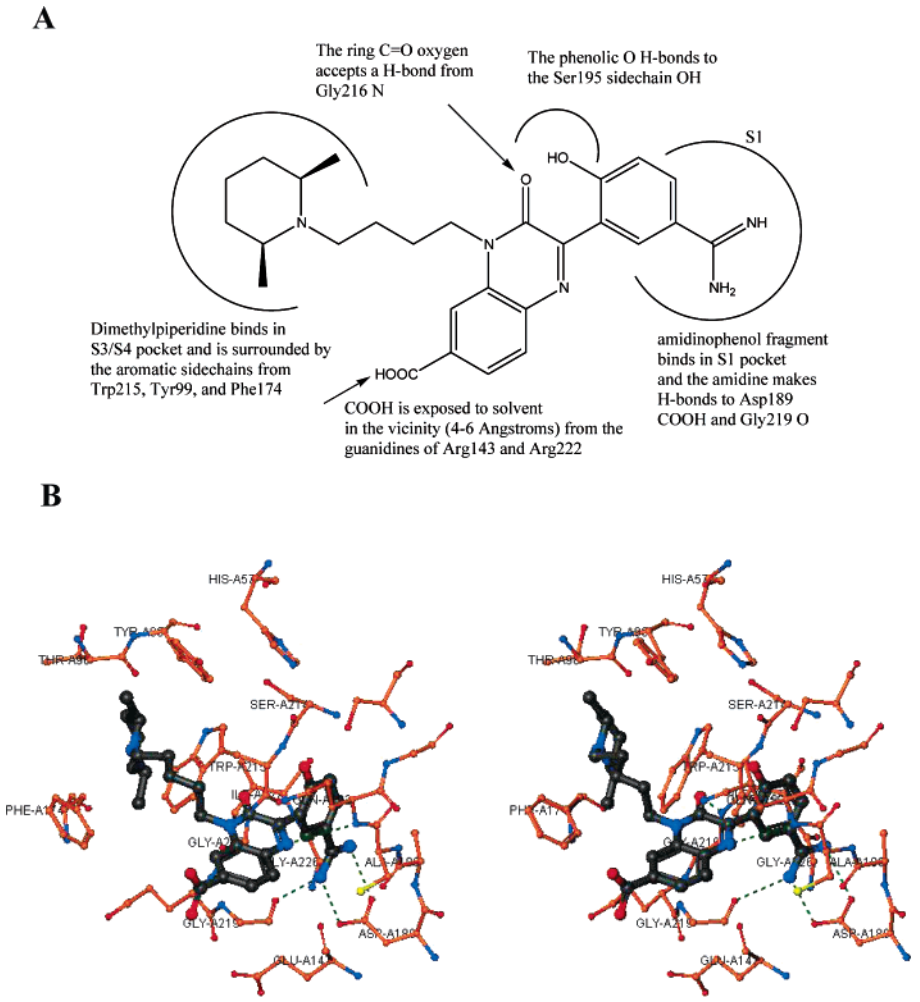


FIGURE 6: The structure of PD0313052 in factor Xa. (A) The chemical structure of PD0313052 is indicated with the appropriate contacts observed in the factor Xa/PD0313052 co-crystal. (B) Stereodiagram of the structure of PD0313052 in the active site of human factor Xa. For structure solution details please see Table 1. The solution structure had an *R*-factor of 0.2, an *R*-free of 0.25, and a resolution of 2.0 Å. The appropriate hydrogen bonding distances are illustrated in green.

of the protease toward large macromolecular substrates is lost following prothrombinase assembly. Attempts to explain substrate specificity of factor Xa from analyses of the active site of the protease based upon the crystal structure have proven problematic. Recent studies have suggested that the high degree of macromolecular substrate specificity of prothrombinase appears to be directed to exosites of the protease that provide unique binding sites for prothrombin recognition (*10*, *11*, *39*). The initial binding of prothrombin to these sites arranges the scissile bonds of prothrombin to promote the two cleavage events necessary for activation to thrombin (*10*). Additionally, the results presented in this study correlate well with the lack of increase in the catalytic activity of factor Xa toward synthetic substrates following its incorporation into prothrombinase (*15*, *40*).

Noteworthy reports from other groups have suggested that some minor alterations at the active site of the human protease may exist following its binding to other members of prothrombinase. The incorporation of Oregon Green$_{488}$ tethered to an active site binding peptide that interacts with the active site of factor Xa demonstrates measurable alterations in the surrounding chemical environment of factor Xa following association with factor Va. The current study illustrates an active site probe that, unlike OG$_{488}$, was specifically designed to interact with the residues deep within the active site of factor Xa in such a way as to position itself securely within the active site pocket of the protease.

While overall explanation of factor Xa function requires analysis of the protease following its incorporation into prothrombinase due to the heavily exosite driven mechanisms of action, the current study suggests that direct inhibition of prothrombinase by small active site binding molecules may be estimated on the basis of the mechanisms of inhibition of factor Xa in solution (*41*, *42*). However, it appears that compounds that are lipophilic may be less potent in prothrombinase compared to factor Xa perhaps due to lipid binding reducing the drug concentration available for inhibition (*42*).

Furthermore, the apparent loss of potency of PD0313052 for prothrombinase that was observed in a plasma based assay system cannot be fully explained by high levels of plasma protein binding by this compound. PD0313052 demonstrates 15.2% protein binding in human plasma (unpublished results), which cannot account for the nearly 1000-fold decrease in effective concentration. Furthermore, as PD0313052 demonstrates greater than 1000-fold selectivity for factor Xa over other serine proteases (data not shown), the difference in observed potency is most likely due to characteristics of the comparative assays. The thrombin generation assay system is not suitable to identifying kinetic

characteristics of inhibitors as there are many substrates for thrombin in the plasma with which the fluorescent substrate must compete and the concentration of the target enzyme is not a constant. However, the affinity of PD0313052 for prothrombinase in plasma as measured by assays of thrombin generation correlate well with IC_{50} values obtained in vivo (unpublished results). Additionally, similar differences in potency of direct factor Xa inhibitors in measurements of tripeptide turnover versus inhibition of prothrombinase in a plasma based system have been previously observed, suggesting that this property is not unique to PD0313052 (*43*). Thus, target inhibitor concentrations may be best selected for use in vivo from studies involving direct measurements of inhibition in a plasma based system with the direct measurements of prothrombinase inhibition.

The data presented in the current study kinetically characterizes the inhibition of both factor Xa and prothrombinase by the highly selective compound PD0313052 and further identifies specific interactions within the active site pocket of factor Xa that may be maintained during prothrombinase assembly. These results further the understanding of the regulation of factor Xa function and should provide for significant future benefits to drug design directed toward this unique protease.

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