Chemistry & Biology, Vol. 11, 1413–1422, October, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.08.006

Disruption of Protein-Membrane Binding and Identification of Small-Molecule Inhibitors of Coagulation Factor VIII

P. Clint Spiegel,[1,3] Shari M. Kaiser,[2,3] Julian A. Simon,[4,5] and Barry L. Stoddard[3,*]

[1]Graduate Program in Biomolecular Structure and Design
[2]Graduate Program in Molecular and Cellular Biology
University of Washington
Seattle, Washington 98195
[3]Division of Basic Sciences
Mailstop A3-025
[4]Division of Clinical Research
[5]Division of Human Biology
Mailstop D2-100
1100 Fairview Avenue, North
Fred Hutchinson Cancer Research Center
Seattle, Washington 98109

Summary

Factor VIII is a critical member of the blood coagulation cascade. It binds to the membrane surfaces of activated platelets at the site of vascular injury via a highly specific interaction between factor VIII's carboxy-terminal C2 domain and their phosphatidylserine-rich lipid bilayer. We have identified small-molecule inhibitors of factor VIII's membrane binding activity that have IC_{50} values as low as 2.5 μM. This interaction is approximately 10^3-fold tighter than that of free o-phospho-L-serine. These compounds also inhibit factor VIII-dependent activation of factor X, indicating that disruption of membrane lipid binding leads to inhibition of the intrinsic coagulation pathway. The tightest binding inhibitor is specific and does not prevent membrane binding by the closely related coagulation factor V. These results indicate that this and related compounds may be used as leads to develop novel antithrombotic agents.

Introduction

High-throughput screening (HTS) strategies are commonly employed to discover small molecules that inhibit potential drug targets [1, 2]. Most of the current targets for drug discovery fall into two categories: G protein-coupled receptors (GPCRs, 45%) and enzyme active sites (28%) [3, 4]. Additional forms of molecular sites and interactions, such as enzyme allosteric sites [5, 6] and protein-protein complexes [7], are also under scrutiny for inhibitor development. To date, there have been no published reports indicating the use of HTS to inhibit protein cofactor/lipid membrane interactions. This is due primarily to the nonspecific nature of many such binding processes and the limited number of structural studies of peripheral binding of proteins to membrane surfaces. However, highly specific protein cofactor/lipid membrane interactions are essential in many biological pathways and processes, such as the blood coagulation cascade [8]. Protein/membrane interactions localize critical protein factors and their associated activities both spatially and temporally, and they can be tightly regulated through the transient presentation of unique membrane compositions on cell surfaces that are then recognized and bound by specific protein ligands [9].

The interaction of many of the coagulation proteins with the membrane surfaces of activated platelets (Figure 1) at the site of a vascular injury leads to a dramatic increase in their activity [10–12]. In the absence of these membrane surfaces (which contain a significant percentage of phosphatidylserine [PS], as well as phosphatidylethanolamine [PE] and phosphatidylcholine [PC]), most coagulation enzymes exhibit negligible activity on a biologically relevant time scale [8]. The serine proteases involved in blood coagulation interact with these platelet surfaces either through highly conserved domains containing γ-carboxyglutamic acid (Gla) residues, and/or via recruitment into complexes with specific platelet binding protein cofactors (either factor V or factor VIII) [10, 11]. These latter proteins associate with the membrane surfaces of activated platelets via highly homologous carboxy-terminal domains (termed the C1 and C2 domains). These domains are members of the discoidin protein family, a diverse group of related protein domains that mediates a wide variety of protein binding interactions [13]. Disruption of the membrane association of the procoagulant clotting factors V and/or VIII results in failure to maintain proper hemostasis [14, 15]. The presence of exposed PS in the activated platelet membranes is crucial for binding and function of these proteins.

Thromboembolic diseases are a major cause of mortality worldwide [16–18]. Traditional antithrombotic treatments mostly consist of low-molecular weight heparins as well as inhibitors of vitamin K epoxide reductase (VKOR), such as coumarin [19, 20]. Both of these anticoagulant families display a range of undesirable clinical side effects stemming from narrow dose-response windows and/or nonspecific target interactions, thereby imparting risks of side effects that make them problematic for long-term use [18]. Safer and more effective treatments for various thromboembolic diseases are therefore desired [17]. Many of the anticoagulation drug candidates under current development are specific inhibitors of one of the serine proteases (particularly factor VIIa, factor Xa, and thrombin) from the cascade (Figure 1). These enzymes represent three of approximately 176 unique serine proteases encoded within the human genome [21].

Recently, antithrombotic "proof of principle" experiments have been directed against factor VIII, which recruits one of the serine proteases from the coagulation cascade (factor IXa) into an active complex on platelet surfaces (Figure 1). A monoclonal antibody, mAb-LE2E9, partially inactivates factor VIII through binding to its C1 and C2 domains. A recombinant form of this antibody

*Correspondence: bstoddar@fhcrc.org

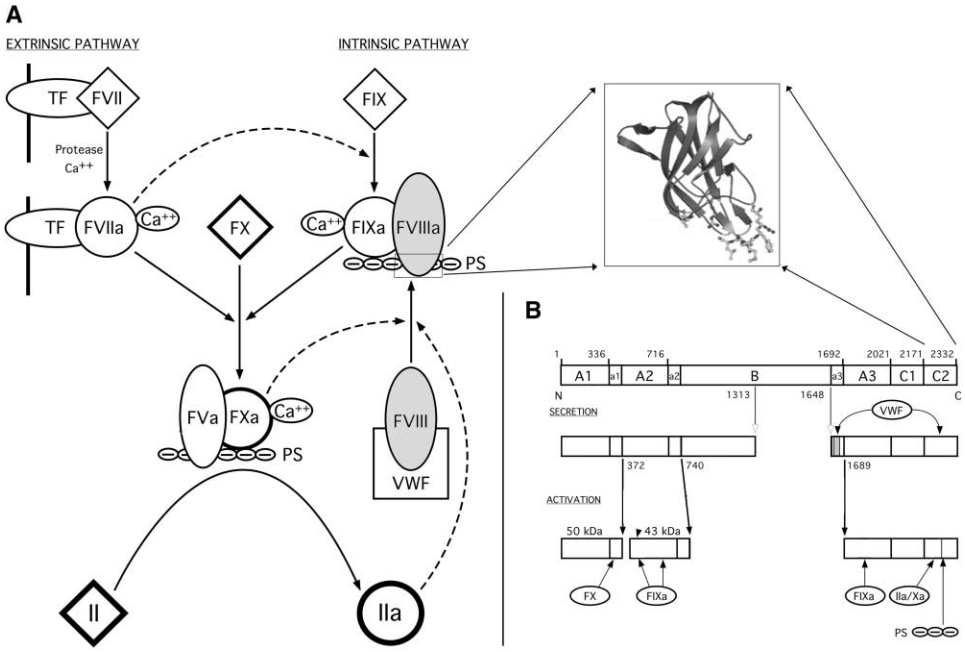


Figure 1. Factor VIII and the Coagulation Cascade

(A) The blood coagulation cascade consists of two pathways (extrinsic and intrinsic) that are initiated by the exposure of tissue factor (TF) or phosphatidylserine (PS) groups of activated platelet membranes to circulating protein factors, respectively. Factor VIII is a large plasma glycoprotein that acts as an initiator and regulator of the intrinsic pathway [44]. Upon proteolytic activation by either factor Xa or thrombin, activated factor VIII (FVIIIa) dissociates from von Willebrand factor (VWF), associates with the factor IXa serine protease, and directs the localization of the resulting complex to the membrane surface of activated platelets, via an interaction with its C-terminal C2 domain (structure in inset) [45]. The membrane-bound factor VIIIa/factor IXa complex functions to proteolytically activate factor X [12], which then activates thrombin (factor II).

(B) Domain structure of factor VIII. Factor VIII is synthesized as a single polypeptide chain of 2,332 residues [11]. Based on sequence homology, factor VIII has the domain structure A1-A2-B-A3-C1-C2, where the A domains are homologous to the copper binding protein ceruloplasmin, the C domains are homologous to the discoidin fold family (structure in inset), and the unique B domain has no known homologs [46–48]. Membrane association is primarily accomplished through the C2 domain; its deletion completely abrogates binding of factor VIII to platelet surfaces [49]. Crystal structures of the factor VIII C2 domain (inset) indicate its probable membrane binding surface [30, 31].

was tested in a murine venous thrombosis model and demonstrated potent antithrombotic properties without bleeding complications [22, 23].

In vivo data demonstrating that inhibition of factor VIII cofactor function leads to reduction thrombosis, as well as biochemical studies that indicate the existence of a defined membrane binding surface on the factor VIII C2 domain, imply that factor VIII might be a viable drug target for the development of novel anticoagulants [17]. Additionally, factor VIII has one of the shortest half-lives ($<$10 hr) and lowest serum concentrations (0.3 nM) in circulation of any of the coagulation factors, increasing its attractiveness as a drug target [12].

We have devised an enzyme-linked immunoadsorbent assay (ELISA)-based, high-throughput screen to identify inhibitors of the factor VIII C2 domain's membrane binding activity. Starting with a screen of 10,000 structurally diverse small molecules, we isolated compounds that inhibit the ability of the factor VIII C2 domain, as well as the full-length protein, to bind phospholipid surfaces similar in composition to activated platelet membranes. Improved analogs of the initial hits were selected from an expanded chemical library consisting of over one million compounds. Inhibitors of factor VIII membrane binding exhibit a dose-dependent inhibition of the intrinsic blood coagulation pathway, as measured by reduced activation of the factor X serine protease. The most potent of these compounds were shown to bind directly to the C2 domain, and to not interact nonspecifically with the membranes themselves, or with either the closely related coagulation factor V or the unrelated membrane binding enzyme phospholipase A2.

Results

Purification and Characterization of the Recombinant Factor VIII C2 Domain

The factor VIII C2 domain consists of residues Ser2173 to Tyr2332 and is the domain responsible for binding to PS-containing membrane surfaces of activated platelets (Figure 1). Purified samples of bacterially expressed, recombinant C2 domain (Figure 2A) were analyzed by circular dichroism (CD) and MALDI mass spectrometry to determine that the protein was properly folded and of the correct mass (18.6 kDa). The C2 domain was characterized in a PS-based ELISA experiment and was shown to bind specifically to immobilized lipid surfaces containing PS (Figure 2A). Similar experiments against membranes containing PC resulted in negligible binding. To indicate whether the C2-PS interaction could be spe-

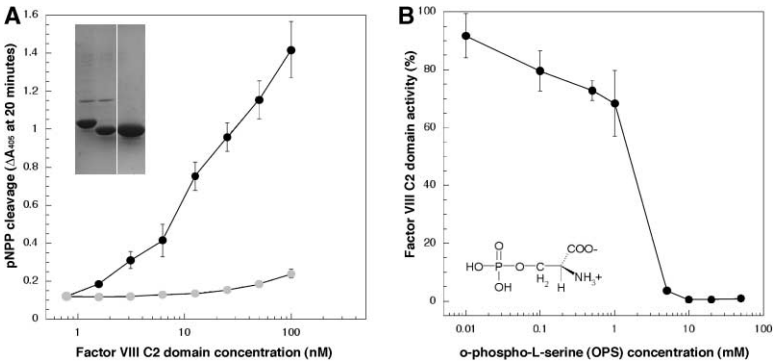


Figure 2. Characterization of Recombinant Factor VIII C2 Domain

(A) Membrane binding ELISA for the C2 domain. The C2 domain binds specifically to the PS surface, while it only binds negligible amounts to PC surfaces (dark circle: PS surface, light circle: PC surface). The inset is an SDS-PAGE gel showing the purification of recombinant C2 domain using a bacterial expression system.

(B) Titration of O-phosphatidylserine (OPS; chemical structure shown in inset) with the C2 domain in membrane binding ELISA.

cifically disrupted, we titrated the soluble headgroup of PS (o-phospho-L-serine) with the C2 domain prior to incubation with the PS-coated plates. As expected, we observed a dose-dependent inhibition of PS association, yielding an IC_{50} value of approximately 2 mM (Figure 2B).

High-Throughput Screening and Validation of Hits

To screen and identify compounds that inhibit the membrane association of the factor VIII C2 domain, we modified the ELISA-based assay described above, preincubating the C2 domain with a set of compounds prior to its addition to PS-immobilized plates. The Chembridge DIVERSet 10,000 compound library was screened in groups of 5 compounds per well. The entire screen was performed in duplicate with positive and negative controls on each plate to decrease the hit rate due to false positives. Top scoring hits were subsequently deconvoluted to determine the identity of each individual inhibitory compound from the initial mixtures. The screen resulted in ten compounds that inhibit the C2-PS interaction completely at an initial concentration of 160 μM (Figure 3).

The IC_{50} values of the ten best compounds from the screen were determined by titrating variable concentrations of compounds against the C2 domain in the membrane binding assay (Figure 4). The IC_{50} values for these compounds span a range of approximately 5–60 μM. Of these hits, the more weakly inhibiting compounds display few structural and/or physicochemical similarities. However, the two hits with the lowest IC_{50} concentration values (compound numbers 3 and 4 in Figures 3 and 4) are related, both containing a tricyclic N-arylbromofurfurylidine-thiazolidinedione core.

Chemical Variation and Analysis of Inhibitor Hits

Commercially available compounds from the extended Chembridge collection that were structurally similar to hits 3, 4, and 9 were analyzed to further test the structure-activity relationship of their inhibitory properties. Analogs of the initial hits that exhibited the strongest inhibition of C2 domain membrane binding are shown in Figure 5A. In the case of compounds 3 and 4, there is a strong correlation between patterns of substitutions on the furan ring and inhibitory activity. When the bromine atom is substituted with either an iodine atom or another halogenated, aromatic six-membered ring (compounds 31, 32, and 41), the IC_{50} is lower (i.e., tighter binding and stronger inhibition). Alternatively, when the bromine atom is replaced with a hydrogen atom, inhibitor activity is significantly reduced (structure not shown). Variations in the phenyl moiety of compound 9 indicate

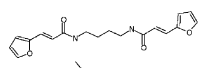
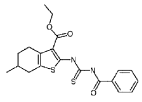
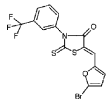
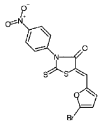
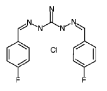
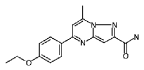
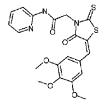
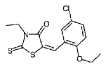
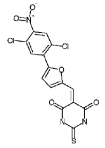
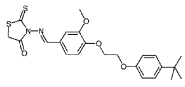
Compound	Structure	Chembridge #	IC_{50} (μM)
1		5247489	56.1±4.9
2		5523453	27.7±5.4
3		5737176	6.7±0.6
4		5739991	4.6±0.7
5		5924029	24.5±3.8
6		6072484	26.8±2.3
7		6126765	11.9±1.2
8		6168356	49.6±4.3
9		6190013	8.5±1.2
10		6209549	12.4±1.7

Figure 3. Chemical Structures of the Ten Best Hits

Each of the chemical structures with their corresponding IC_{50} values based on the membrane binding ELISA are grouped into three categories: weak (compounds 1, 2, 5, 6, and 8 had an $IC_{50} > 20$ μM), moderate (compounds 7 and 10 had an IC_{50} between 10 and 20 μM), and strong (compounds 3, 4, and 9 had an $IC_{50} < 10$ μM).

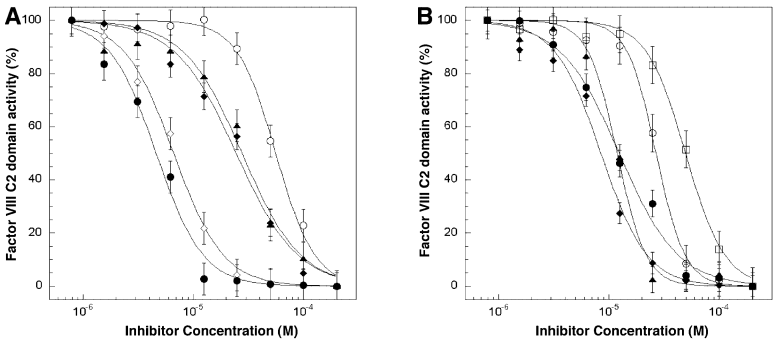


Figure 4. Titration Curves for the Ten Best Hits in the PS-Based ELISA

(A) Compounds 1–5 in the PS-based ELISA using the C2 domain (open circle: 1, closed triangle: 2, open diamond: 3, closed circle: 4, closed diamond: 5).

(B) Compounds 6–10 in the PS-based ELISA using the C2 domain (open circle: 6, closed triangle: 7, open square: 8, closed diamond: 9, closed circle: 10). Binding curves were calculated by nonlinear regression with the program GraphPad Prism.

that altering the position of either the nitro group or the chloride atoms does not significantly affect the activity of this class of compounds. Of the analogs tested, compound 32 in our screen (Figure 5A; Chembridge #8047577) displayed the lowest IC_{50} for inhibition of membrane binding by the factor VIII C2 domain (2.4 μM). This compound contains a dichlorophenyl substitution for the furanyl bromine atom in compound 3 from the original screen.

Control Experiments: The Inhibitors Act Specifically on Factor VIII

To validate that the most potent compounds in this study were specifically binding to the factor VIII C2 domain and thereby inhibiting its membrane binding interaction, six control experiments were conducted. First, the ability of the immobilized membranes to support protein binding in the ELISA assay was shown to be unimpaired after their direct incubation with the individual compounds and a secondary wash step (data not shown). Second, the inhibitors were also shown to inhibit factor VIII-dependent activation of factor X, under conditions in which phospholipids were in vast excess of inhibitors (see results below). Third, the best inhibitors from the study were shown to bind directly to the C2 domain by fluorescence spectroscopy, with equilibrium dissociation constants (K_D) that are similar to the observed IC_{50} values from membrane binding inhibition curves (39.9, 32.8, and 28.8 μM for compounds 3, 32, and 9, respectively). Fourth, to validate that the best compounds were specifically blocking the membrane binding surface and activity of factor VIII rather than simply inducing protein aggregation, the solution behavior of the protein was assayed by size-exclusion chromatography and dynamic light scattering after incubation with compounds and was found to be unchanged (data not shown). Finally, the best inhibitors of factor VIII membrane binding were tested and shown to be incapable of blocking membrane binding activity of either the closely related homolog factor V (which displays 40% sequence identify and identical phospholipid binding specificity with factor VIII) (Figure 5B) or the unrelated membrane binding enzyme cytosolic phospholipase A2 (cPLA2). This latter protein binds neutral phospholipid surfaces with a structural membrane binding region that also is termed a "C2" domain, but is completely unrelated to the C2 domain of factor VIII [24].

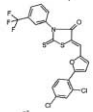
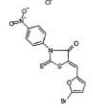
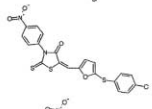
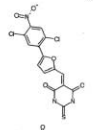
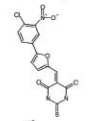
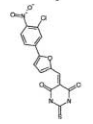
Inhibition Studies with Recombinant, Full-Length Factor VIII

To determine whether the inhibitory activity of these compounds toward the factor VIII C2 domain corresponds to similar inhibition of full-length factor VIII, we performed titrations of compounds with the PS-based ELISA experiment using a recombinant, full-length factor VIIIa heterodimer [25]. The most active compounds from C2 domain inhibition assays (3, 31, 32, 4, 41, 9, 91, and 92) were tested to determine their corresponding IC_{50} values. For those compounds related to hits 3 and 4 in the original screen (which are themselves closely related to each other as described above), the IC_{50} values for inhibition of membrane binding by either the C2 domain or the full-length factor VIIIa heterodimer are similar. Compound 32, which exhibits the most effective inhibition of the C2 domain, is also the best inhibitor of factor VIIIa (Figure 5A). The third hit to be varied and tested (compound 9 and its cousins) displays lower correlations between the IC_{50} values against the free C2 domain and the full-length factor VIIIa construct. Conserved patterns of physicochemical properties that appear important for inhibitor activity are most consistent for those compounds that exhibit the tightest binding in membrane binding inhibition assays.

Inhibition of Factor X Activation

The screens described above were developed and conducted based on the hypothesis that inhibitors of factor VIII membrane binding should also inhibit the ability of factor VIII to act as an activating cofactor in the intrinsic coagulation pathway. In order to address this hypothesis, we conducted an in vitro assay to monitor the rate of factor VIII-dependent activation of factor X as a function of the presence and concentration of the small-molecule inhibitors of factor VIII lipid binding described above. In the presence of calcium ions and phospholipids containing PS, factor X is activated by factor IXa (a process sometimes called the tenase reaction). This reaction is greatly enhanced by the presence of factor VIII. Utilizing a chromogenic substrate that is specific for factor Xa, one can measure the rate of factor X activation to factor Xa. Antibodies specific to the C2 domain of factor VIII have been shown previously to inhibit the ability of factor X to become activated. The strongest inhibitors described above were titrated (20–1.25 μM) against recombinant factor VIII in this assay to determine the IC_{50} values for the inhibition of factor

A

Compound	Structure	Chembridge #	IC_{50}(C2)	IC_{50}(FVIII)	IC_{50} (FXase)
3		5737176	6.66±0.60	9.25±1.10	8.5 μM
31		5738875	5.65±1.10	6.83±0.74	5.0 μM
32		8047577	2.44±0.29	2.56±0.45	2.1 μM
4		5739991	4.63±0.70	10.4±1.30	11.7 μM
41		6789536	5.85±0.78	4.81±0.45	5.6 μM
9		6190013	8.48±1.20	2.11±0.31	7.5 μM
91		8048422	4.17±0.59	3.89±0.29	6.7 μM
92		8056354	6.29±0.48	6.51±0.85	6.8 μM

B

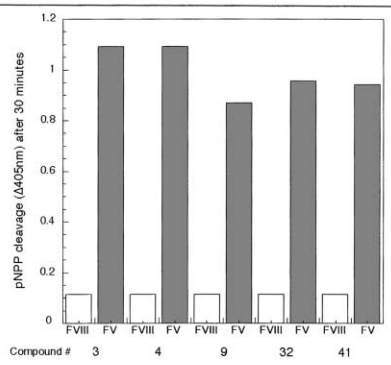


Figure 5. Chemical Structures of Variations to Compounds 3, 4, and 9

(A) Chemical structures of compounds 3, 31, 32, 4, 41, 9, 91, and 92 are shown with their associated IC_{50} values for the inhibition of the C2 domain and factor VIII ELISA and for the inhibition of the factor X activation assay. Structural variations and associated IC_{50} values indicate a structure-activity relationship for the mode of binding to the C2 domain. Substitutions at the bromine atom in compounds similar to 3 and 4 have effects of the potency. Larger or more hydrophobic substitutions increase the inhibitory activity.

(B) Relative inhibition of factor VIII and factor V by various inhibitors from the panel above, all measured at 20 μM concentration.

X activation. The titrations for compounds 3, 31, 32, 9, 91, and 92 approach complete inhibition of the factor X activation at 20 μM, again with IC_{50} values that are similar to those measured for inhibition of factor VIIIa membrane binding (Figures 5 and 6). Of these compounds, number 32 (the most effective inhibitor of membrane binding) is also the most effective inhibitor of factor X activation, with an IC_{50} value of 2.1 μM. In contrast, compounds 4 and 41 never approach complete inhibition of the activation of factor X, only inhibiting at approximately 35%–40% at 20 μM compound concentration. To ensure that the compounds were not acting directly on factor X in this assay, a control was done for each in which the reaction mix was spiked with an excess of preactivated factor Xa (1 μg/ml). All compounds had little or no effect on the intrinsic ability of factor Xa to cleave its chromogenic substrate.

Finally, a panel of five compounds (3, 4, 9, 41, and 32) were tested for their ability to inhibit the factor VIII-dependent activation of factor X by using platelet-enriched human serum as a source of membranes, to test whether their inhibitory properties would be re-

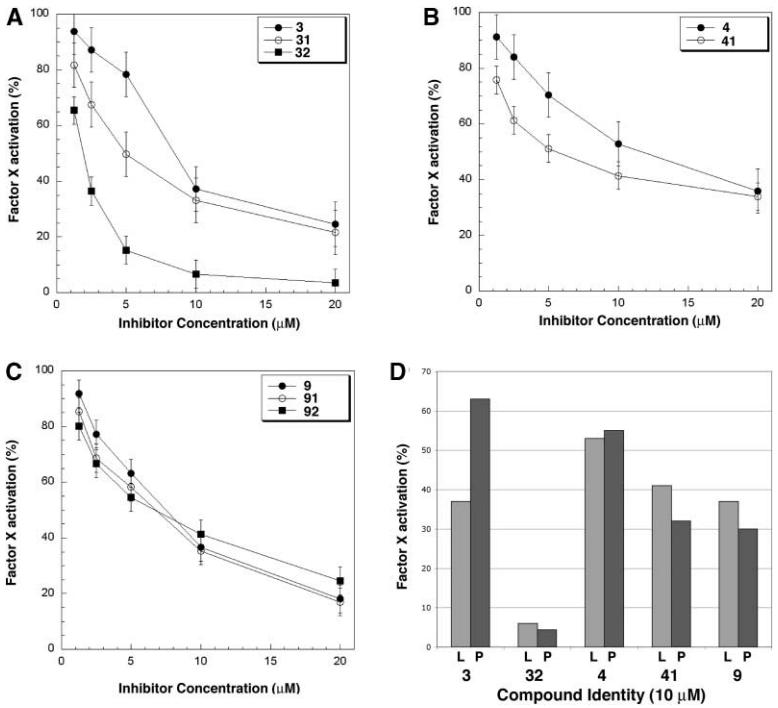


Figure 6. Inhibitory Effect of Compounds on Factor X Activation

(A) Titrations of compounds 3 (closed circle), 31 (open circle), and 32 (square).

(B) Titrations of compounds 4 (closed circle) and 41 (open circle).

(C) Titrations of compounds 9 (closed circle), 91 (open circle), and 92 (square).

(D) Relative inhibition of factor X activation by factor VIII.

(E) Relative inhibition of factor X activation in the presence of purified lipids (L) and platelet-rich plasma (P). Activation of factor X in the absence of added compounds, as described in the Experimental Procedures, gives a signal in the assay defined as 100%. All compounds demonstrate inhibition of factor VIII activity in this assay. At 10 μM concentrations, one compound [3] displays a reduced potency in the presence of platelet-rich plasma; the remainder display similar inhibition properties. Compound 32 is potent under either condition.

duced in the presence of physiological plasma components and when using platelet surfaces as a substrate, rather than purified lipids. These experiments were performed by using the protocol of Ahmad et al. [26], with the exception that platelets were not gel purified away from serum components, but rather were added as platelet-rich plasma in order to more closely mimic in vivo circulation. Platelets were counted by using a hemocytometer; their concentration in the experiment was 2×10^7/ml. At 10 μM concentrations, all compounds demonstrated inhibition of the factor X activation in the presence of platelet-rich serum. Compound 3 exhibited a signficantly reduced activity, while the remaining four retained potency. As in previous studies with purified components, compound 32 was the most effective, demonstrating almost complete inhibition of factor X activation. (Figure 6D).

Discussion

The basic principles of this work are straightforward. Factor VIII binds to specific anionic membrane surfaces of activated platelets, at the site of a vascular injury, and then acts to recruit additional activated clotting factors (particularly factor IXa) to that location (Figure 1). Factor VIII binds to these membrane surfaces via interactions between its C-terminal C2 domain and the platelets' PS-rich phospholipid bilayer. We predicted that inhibition of this interaction by a small molecule would lead to inhibition of steps in the coagulation cascade that are dependent on factor VIII, such as activation of the serine protease factor X. We are able to identify inhibitors of the factor VIII-membrane interaction and demonstrated that such compounds can prevent factor VIII from participating in the activation of factor X.

Factor VIII is an attractive drug target for at least two reasons. First, it possesses the lowest plasma half-life and concentration (10 hr and 0.3 nM, respectively) of all the coagulation factors in the intrinsic pathway, corresponding to a lower target concentration for therapeutic inhibition [12]. Second, it utilizes a structural protein fold for membrane binding (a discoidin domain) that is far more rare in the human genome and also more highly diversified at the level of primary sequence and tertiary structure than is the serine protease family (the source of anticoagulant targets thrombin, factor VIIa and factor Xa) [13, 21]. The structural conservation of serine proteases (176 significant, unique representatives in the human genome identifiable by BLAST searches, compared to 23 identifiable homologs of the factor VIII C2 discoidin domain) is a well-documented source of undesirable nonspecific pharmacological effects in clinical applications.

Based on the structural and biochemical data describing the mechanism of membrane binding for the factor VIII C2 domain [27–29], it seemed plausible that potent inhibitors can be developed that target a specific region of the C2 domain. The crystal structures of the C2 domain alone and in complex with an inhibitory antibody derived from a hemophilia A patient indicate a binding cleft at one end of the molecule that consists of solvent-exposed hydrophobic residues and positively charged basic residues [30, 31]. Additional biochemical data suggest that this region is primarily responsible for binding to activated platelet surfaces based on mutational results [27, 29]. Moreover, membrane binding experiments indicate that factor VIII binds preferentially to the L-isomer of PS, further supporting a specific interaction between factor VIII and membrane surfaces on activated platelets [32]. Lastly, mutational data suggest that per-

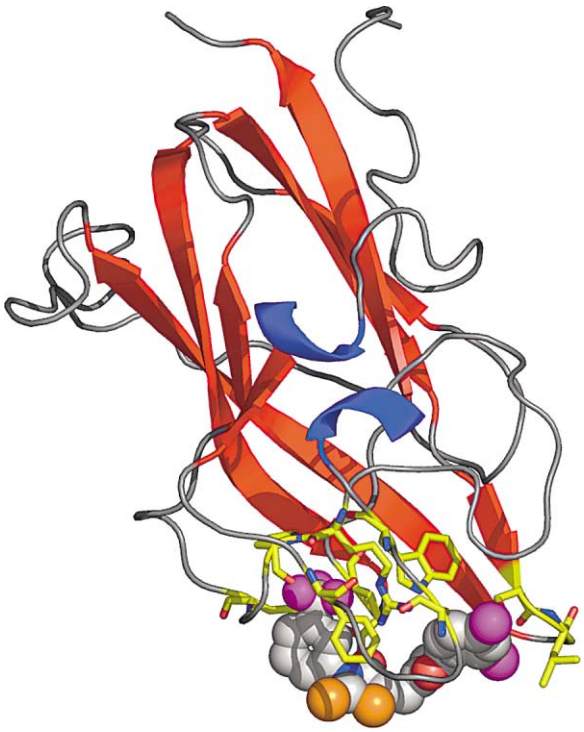


Figure 7. Docked Model of Compound 32 Bound to the Factor VIII C2 Domain

Compound 32 was docked to the membrane binding surface of the C2 domain by using protein AUTODOCK [50]. The conformation shown in the figure corresponds to the best (lowest) calculated energy in a collection of 50 separate runs and also represents a cluster of several independent hits that converged to similar bound conformations from those runs. The interactions between compound and protein domain in this docked model are described in the Discussion.

turbation of the C2 domain's membrane binding surface is associated with deficiencies in factor VIII activity [33].

The observation that the highest affinity inhibitor of factor VIII membrane binding does not prevent similar binding by the closely related factor V provides important confirmation of specificity as well as insight into potential modes of its interaction. Computational docking experiments with compound 32 against the crystallographic model of the factor VIII C2 domain indicate that the compound may bind to a pair of pockets flanked by the hydrophobic β hairpin turns of its membrane binding surface (Figure 7). In this model, the two six-membered phenyl rings are both partially buried in these pockets, with their trifluoro and dichloro substituents in contact with protein backbone and side chain atoms. The entire compound is wrapped around an aromatic side chain (Phe2196) that separates the two pockets. This binding mode agrees well with the effect of chemical substituents on the inhibitory IC_{50} values described above, particularly the contribution of the phenyl rings and their chemical substituents to overall affinity. Two residues in this region that might contact the bound inhibitor (Phe2196 and Ala2201) are poorly conserved in factor V and are instead a lysine and an aspartic acid, respectively. In contrast, surrounding protein side chains that participate in membrane insertion are more strongly conserved.

The combination of these results indicates that the discovery of small molecules against the membrane association of the factor VIII C2 domain could lead to the development of potent antithrombotic therapeutics. Additionally, the use of simple, single domain subunits (such as the C2 domain) of large, multidomain protein complexes (factor VIII) as targets in high- throughput screening (HTS) experiments may lead to more rapid assay development and could circumvent cost prohibitive experiments. In many cases, the development of HTS protocols is often hindered by the ability to produce enough reagents for use in a large-scale assay. The results presented here indicate that inhibitors targeting simple and easily produced subunits can directly translate to function against the entire macromolecular complex.

Although the compounds described in this paper (particularly compound 32, with an in vitro IC_{50} value of 2 μM) display reasonable inhibition of the membrane binding and factor X activation activities of purified factor VIII in the presence of synthetic PS-rich lipids or activated platelets, the compounds in their current form display relatively poor inhibition of factor VIII-dependent procoagulant activity in whole plasma (i.e., against endogenous factor VIII). One possible explanation for this observation is that binding of these compounds is competitive against von Willebrand factor, which is present in whole plasma and the circulation in vast excess of factor VIII, and is thought to bind to factor VIII by interacting with its membrane binding surface [29, 33–38]. If this is true, then the further optimization of the compounds reported here, into inhibitors with low nanomolar affinities, may be necessary before significant in vivo anticoagulant activities are realized.

In conclusion, the use of a competition assay based on disrupting the interaction between a nonenzymatic protein cofactor and its affinity for the hydrophilic headgroup of a specific phospholipid surface leads to a new area for the discovery of small-molecule inhibitors. Our results indicate that, through the use of high-throughput screening techniques, we have determined a novel method for discovering small-molecule inhibitors of protein/membrane interactions, leading to the identification of a class of compounds that display inhibition of the procoagulant function of factor VIII.

Significance

To the best of our knowledge, this study provides the first example of using drug discovery methods, including high-throughput compound library screening, to inhibit a specific protein-membrane binding interaction. While such a strategy may be limited to protein-membrane interactions that are highly specific, it represents a significant departure from the development of enzyme inhibitors and/or receptor antagonists. The interaction targeted in this study (the binding of factor VIII to the surface of activated platelets) represents a novel strategy for the design of small compounds with anticoagulant properties. The development of a class

of anticoagulants that do not target serine proteases such as factor X may provide a useful adjunct to the current classes of compounds now under study. A significant question remaining to be answered is whether further improvement in affinity and specificity over that reported in this paper can be realized, and whether those improvements will translate into significant anticoagulant activity in vivo.

Experimental Procedures

Reagents

Monoclonal antibody (mAb) ESH-8 was purchased from American Diagnostica, Inc., and an Fc-specific, goat anti-mouse IgG that is alkaline phosphatase (AP)-conjugated was purchased from Sigma. Maxisorb plates (96-well) were purchased from Nunc, o-phosphatidyl-L-serine was purchased from Avanti Polar Lipids, Inc., and bovine serum albumin (fraction V powder) and p-nitrophenyl phosphate (pNPP) were purchased from Sigma. Recombinant, full-length factor VIIIa heterodimer was a generous gift from Y.H. Kim (Suwon, South Korea). The COAMATIC factor X activation assay kit is a product of Chromogenix that was purchased through Diapharma Group, Inc. The DIVERSet compound library and subsequent individual variants of initial compound hits from that library were purchased from Chembridge Corporation. Factor V and anti-factor V antibody (clone HFV-1) were purchased from American Diagnostica, Inc. Cytosolic phospholipase A2 (cPLA2) was provided by the Michael Gelb lab (University of Washington Department of Chemistry), who also conducted inhibition assays with that enzyme for this study.

Factor VIII C2 Domain Expression/Purification

A construct containing the factor VIII C2 domain (residues 2171–2332) was subcloned into a pET15b plasmid (Novagen) by using the restriction enzymes NdeI and BamHI (New England Biolabs) and transformed into the AD-494 cell line (Novagen) cotransformed with a plasmid encoding for rare tRNAs (RIL, Novagen). Five milliliter cultures were grown overnight from a single colony at 37°C in Luria Broth (LB) medium containing 35 mg/L chloramphenicol, 25 mg/L kanamycin, and 100 mg/L ampicillin. One liter cultures were inoculated with 5 ml overnight cultures and grown to an OD_{600} of 0.8–1.0. C2 domain expression was induced by the addition of 1 mM isopropyl-thio-β-D-galactosidase (IPTG). Inductions were grown for 16–20 hr at 16°C with shaking.

Cells were lysed by sonication in 300 mM NaCl, 20 mM Tris-HCl (pH 7.0), 10 mM imidazole, 0.01% (v/v) Triton X-100, 100 μM phenylmethylsulfonyl fluoride (PMSF), and 2.5% (v/v) glycerol. Lysates were centrifuged at 16,000 rpm in an SS-34 rotor (Sorvall) for 30 min. The supernatant was applied to TALON metal affinity resin (Clontech) at 4°C for 1.5–2.0 hr. The protein-resin slurry was applied to a gravity-flow column. The resin was washed with 20 column volumes of 300 mM NaCl, 20 mM Tris-HCl (pH 7.0), 10 mM imidazole, and 2.5% (v/v) glycerol and subsequently washed with 20 column volumes of 150 mM NaCl, 20 mM Tris-HCl (pH 7.0), 10 mM imidazole, and 2.5% (v/v) glycerol. The C2 domain was eluted in 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 150 mM imidazole, and 2.5% (v/v) glycerol and subsequently dialyzed into 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 2.5% (v/v) glycerol. The histidine tag was subsequently removed with biotinylated thrombin according to the manufacturer's protocol (Novagen). The C2 domain was further purified by using a heparin affinity column (Applied Biosystems). The purified C2 domain was concentrated to 5 mg/ml, flash frozen in liquid nitrogen, and placed at –80°C for storage.

Phosphatidylserine Binding ELISA

The interaction between factor VIII and PS was measured by using an enzyme-linked immunoadsorbent assay (ELISA) that was adapted from previously described methods [39, 40]. Maxisorb plates (96-well) (Nunc) were incubated with 100 μl 5 μg/ml PS dissolved in methanol and dried overnight at room temperature. Plates were then blocked with 200 μl of 2% (w/v) bovine serum albumin in Tris-buffered saline (BSA-TBS). 100 μl factor VIII or its C2 domain (or full-length factor V in control experiments), diluted to 50 nM in BSA-TBS, was then incubated for 60 min at 37°C with shaking. Plates were rinsed three times with TBS between each incubation. Subsequent to the C2 domain incubation, 100 μl mAb ESH-8 diluted 1:1000 in BSA-TBS was added. 100 μl of an alkaline phosphatase (AP)-conjugated goat anti-mouse antibody was then added. Factor VIII and/or the C2 domain that had bound to the plate was detected by the addition of p-nitrophenyl phosphate (pNPP), followed by absorbance measurements at 405 nm with a microplate reader.

Compound Library Screening

The library of 10,000 compounds was diluted from 20 mM stocks in dimethyl sulfoxide (DMSO) to groups of 5 compounds each at 750 μM per compound in water. For high-throughput assay purposes, the C2 domain diluted in TBS was incubated with each mixture of drugs at 200 μM for 30 min at room temperature. BSA was subsequently added to a concentration of 2% (w/v). The resulting mixture was then added to the ELISA plates at the C2 domain incubation step. Subsequent development of binding assays was carried out as described above, and deconvolutions from initial hits and titrations were performed similarly.

Factor X Activation Assay

A commercially available chromogenic assay that measures the rate of factor X activation was used according to the manufacturer's instructions to measure inhibition of the cofactor function of factor VIII [41]. When factor VIII is added to a reaction mixture containing thrombin, factor IXa, excess factor X, calcium, phospholipids, and a chromogenic substrate specific for factor Xa (S-2765), a measure of factor X activation can be detected and is linearly related to factor VIII activity. Full-length, recombinant factor VIIIa heterodimer was diluted to 1 μg/ml in sample buffer (25 mM Tris-HCl [pH 7.9] and 1% [w/v] bovine serum albumin) and incubated with various drug concentrations. The different factor VIII mixtures were then used as a substitute for plasma samples containing the appropriate concentration of factor VIII. Proteolytic cleavage of the chromogenic substrate was measured with a microplate spectrophotometer at an absorbance of 405 nm. Reactions were stopped by the addition of 20% (v/v) glacial acetic acid.

The assay was also performed using platelet-rich plasma (obtained as a gift from the clinical division of the Hutchinson Center) in place of purified phospholipids, in order to determine if the inhibitors retain potency in a system that more closely resembles the blood circulation. After low-speed centrifugation to remove erythrocytes, the platelet count was determined by using a hemocytometer, and platelets were added to a concentration of 2×10^7/ml, corresponding to the platelet concentration typically used in similar assays described by Ahmad et al. [26]. Purified platelets were not used in this assay in order to more closely mimic natural human plasma.

Compound Binding to C2 Domain

Fluorescence binding experiments to demonstrate direct binding of optimal compounds to the C2 domain and thereby determine an equlibrium dissociation constant (K_D) were performed on a Perkin Elmer LS50 B Luminescence spectrometer. The excitation wavelength was 278 nm, with bandwidths of 5 nm for excitation and 5 nm for emission; fluorescence was monitored from 310 nm to 400 nm. Fluorescence titrations were performed by sequentially adding 0.5–5 μl of a concentrated ligand solution to 3000 μl of the C2 domain solution. The fluorescence signal and ligand concentration were adjusted for the effects of dilution. Fluorescence quenching was calculated from $Q = (F - F_0)/F_0$ where F is the intensity of the fluorescent signal corrected for dilution and F_0 is the signal in the absence of ligand. Dissociation constants were calculated by fitting the quenching data to the equation:

$$Q = Qmax/(1 + K_D/[Ligand]), \quad (1)$$

where Qmax is the maximal quenching in the absence of ligand and K_D is the apparent dissociation constant for the ligand.

Phospholipase A2 Membrane Binding and Enzyme Activity Assay

Recombinant cytosolic group IV phospholipase A_2 with a C-terminal 6-histidine tag was prepared as described [42]. The assay with the fluorimetric substrate Glu (the umbeliferryl ester of γ-linolenic acid)

has been described previously [43]. Briefly, assay buffer consists of 50 mM Tris (pH 8.0), 150 mM NaCl, 0.2 mM EGTA, 0.5 mM $CaCl_2$, 30% (v/v) glycerol, and 0.024% (w/v) Triton X-100. A total of 2.8 μl Glu (1 mM in DMSO was added to 1 ml of assay buffer in a fluorescence cuvette. Excitation was at 375 nm, and emission was monitored at 460 nm with stirring at room temperature. The assay was initiated by the addition of 0.48 μg of cytosolic phospholipase A_2, and the emission was followed for 2 min. Inhibitor was added at various concentrations to assay buffer prior to the addition of enzyme.

Acknowledgments

We acknowledge Paul Murphy, Jeff Posakony, and Melisa Nelson (Simon Lab, Fred Hutchinson Cancer Research Center, Division of Clinical Research) for technical assistance with compound screening, Farideh Ghomashchi in the Michael Gelb lab (University of Washington Department of Chemistry) for performing activity assays of phospholipase A2, and other members of the Stoddard, Simon, Strong, and Ferre-D'Amare labs for encouraging discussions and assistance. This work is supported by National Institutes of Health (NIH) R01HL62470 (B.L.S), NIH T32GM08268 (P.C.S), a National Science Foundation pregraduate fellowship (S.M.K), and NIH R01CA90563 (J.A.S).

Received: April 29, 2004
Revised: August 3, 2004
Accepted: August 4, 2004
Published: October 15, 2004

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