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Generation and characterization of aptamers targeting factor XIa

R. S. Woodruff^{*,‡}, I. Ivanov[†], I.M. Verhamme[†], M-f. Sun[†], D. Gailani[†], and B.A Sullenger^{*}

^{*}Department of Surgery, Duke University Medical Center, Durham, NC

[‡]University Program in Genetics and Genomics, Duke University, Durham, NC

[†]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN

Abstract

Background—The plasma protease factor XIa (FXIa) has become a target of interest for therapeutics designed to prevent or treat thrombotic disorders.

Methods—We used a solution-based, directed evolution approach called systematic evolution of ligands by exponential enrichment (SELEX) to isolate RNA aptamers that target the FXIa catalytic domain.

Results—Two aptamers, designated 11.16 and 12.7, were identified that bound to previously identified anion binding and serpin bindings sites on the FXIa catalytic domain. The aptamers were non-competitive inhibitors of FXIa cleavage of a tripeptide chromogenic substrate and of FXIa activation of factor IX. In normal human plasma, aptamer 12.7 significantly prolonged the aPTT clotting time.

Conclusions—The results show that novel inhibitors of FXIa can be prepared using SELEX techniques. RNA aptamers can bind to distinct sites on the FXIa catalytic domain and noncompetitively inhibit FXIa activity towards its primary macromolecular substrate factor IX with different levels of potency. Such compounds can be developed for use as therapeutic inhibitors.

Keywords

RNA aptamers; Factor XIa; Factor IX; Anticoagulant Agents; Blood Coagulation

Correspondence: Dr. B.A. Sullenger, Department of Surgery, Duke University Medical Center, Box 103035, Durham, NC 27710; tel.: (919) 684-6375; fax: (919) 684-6492; bruce.sullenger@duke.edu.

ADDENDUM R.S.W. and I.I. designed and performed research, analyzed data and wrote the manuscript. I.V. analyzed the kinetic data of S-2366 hydrolysis and factor IX activation. M-f.S. prepared and characterized variants of factor XI. D.G. and B.A.S. designed and coordinated research, analyzed data, and wrote the manuscript.

Conflict of interest: Dr. Sullenger reports founding equity from Regado Biosciences/Tobira, outside the submitted work; In addition, Dr. Sullenger has a patent issued broadly relevant to the submitted work. Dr. Gailani reports grants from National Institutes of Health HL58837, HL81326, GM116184, a member of the advisory board of Aronora, grants and personal fees from Bayer, grants and personal fees from Dyax, personal fees from Ionis, personal fees from Merck, personal fees from Novartis, grants and personal fees from Ono, outside the submitted work; In addition, Dr. Gailani has a patent U.S. Patent Application No. 13/140,115 with royalties paid.

INTRODUCTION

Approved antithrombotic drugs target thrombin generation either by inhibiting thrombin and/or factor Xa, or by lowering the plasma levels of the zymogen precursors of these enzymes [1,2]. Because thrombin and factor Xa are central to hemostasis, agents targeting them will inevitably increase bleeding risk. There is interest in developing strategies directed at other enzymes involved in coagulation, with the expectation that they will be safer than inhibitors of thrombin or factor Xa from the standpoint of bleeding. Data from rodent, rabbit and primate models suggest that inhibition of the proteases factor XIa (FXIa) or factor XIIa (FXIIa) reduces occlusive thrombus growth while having relatively small effects on hemostasis [3-7]. Humans lacking factor XII (FXII), the zymogen of FXIIa, do not bleed abnormally, while individuals deficient in factor XI (FXI), the zymogen of FXIa, have a relatively mild bleeding propensity [8]. There are reasons to suspect that FXIa may be preferable to FXIIa as an antithrombotic target. Antibodies that neutralize FXI have a greater effect in a primate thrombosis model than do antibodies inhibiting FXII [9-12]. FXI appears to contribute to stroke and venous thromboembolism (VTE), and perhaps myocardial infarction, in humans, while data supporting a role for FXII in these disorders is weak [3]. A recent phase 2 study demonstrated that reduction of plasma FXI could prevent venous thromboembolism with minimal disturbance of hemostasis in humans undergoing knee replacement [13].

Human FXI is a 160-kDa protease that circulates as a disulfide-linked dimer of identical 80-kDa subunits [14,15]. FXIa contributes to thrombin generation primarily by activating factor IX. Each FXIa subunit contains a 45-kDa heavy chain with four ~90 amino acid domains (designated A1 to A4), and a 35-kDa trypsin-like catalytic domain. Regulation of FXI activation and FXIa activity involves anion-binding sites (ABSs) on the A3 and catalytic domains. Polyphosphates, which are polymers of inorganic phosphate released from activated platelets, bind to FXI ABSs and accelerate zymogen activation [16-18]. Binding of heparin and serpins to the FXIa ABSs results in inhibition of FXIa [19-21].

Anticoagulant aptamers have been developed that target several proteases involved in blood coagulation [22-26]. Aptamers are short, single-stranded oligonucleotides that bind over large surface areas on a target protein. Those that act as anticoagulants usually block specific macromolecular interactions. Here, we describe the isolation and characterization of a library of RNA-based aptamers targeting FXIa. Two aptamers were identified that bind to ABSs on the FXIa catalytic domain, inhibiting FXIa cleavage of a tripeptide chromogenic substrate, and inhibiting FXIa activation of FIX.

MATERIALS AND METHODS

Materials

SELEX DNA templates 5'-TCGGGCGAGTCGTCTG-N₄₀-CCGCATCGTCCTCCCTA-3' were from Oligos etc. (Wilsonville, OR). 5' and 3' SELEX primer sequences, 5'-GGGGGAATTCTAATACGACTCACTATAGGGAGGACGATGCGG-3' and 5'-TCGGGCGAGTCGTCTG-3', respectively, were from Integrated DNA Technologies (Coralville, IA). 2'-fluoro modified cytidine and uridine were from Trilink BioTechnology

(San Diego). FXI, FXIa, factors IX and IXa β were from Haematologic Technologies (Essex Junction, VT). FXIIa was from Enzyme Research Lab (South Bend, IN). Normal pooled plasma was from George King Biomed (Overland Park, Kansas). PTT-A Reagent for partial thromboplastin time (aPTT) assays was from Diagnostica Stago (Parsippany, NJ). AMV-RT enzyme was from Roche Applied Science (Indianapolis). Bovine serum albumin (BSA) was from EMD Chemicals (Gibbstown, NJ), ethylene glycol was from Sigma-Aldrich (St. Louis) and lima bean trypsin inhibitor was from USB Corporation (Cleveland). Pefachrome FXIa 3107 was from Centerchem, Inc., (Norwalk, CT) and S-2366 was from DiaPharma Group, Inc. (West Chester Township, OH). Kinetic assays were performed in 96-well flat bottom microtiter plates (Corning, Corning, NY).

SELEX (Systematic Evolution of Ligands by Exponential Enrichment)

Solution based SELEX was performed as described [22]. The sequence of the starting RNA template library (termed Sel-3) was 5'-GGGAGGACGAUGCGG-N40-CAGACGACUCGCCCCGA-3', where N40 is a 40 nucleotide randomized region, where equal molar amounts of the four standard nucleobases are included, and C and U are 2'-flourocytidine and 2'-flourouridine ribonucleotides, respectively. This RNA library was transcribed using a modified T7 RNA polymerase (T7 RNA polymerase Y639F) that incorporates 2'-flourocytidine triphosphate and 2'-flourouridine triphosphates [27]. Selection rounds were performed by incubating the starting aptamer library Sel-3 with FXIa and passing the mixture through a 25-mm Protran BA 85 0.45 μ m nitrocellulose filter membrane (Whatman, Piscataway, NJ) to separate bound and unbound RNA. Bound RNA was extracted and amplified by RT-PCR. For rounds 1 to 6, 1 nmol RNA was incubated with FXIa in low salt buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM CaCl₂, and 0.01 % BSA). For rounds 7 to 14, 1 nmol RNA was incubated with FXIa in physiologic salt buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and 0.01% BSA). Rounds 11, 12 and 14 were cloned and approximately 30 individual aptamers were sequenced per round, as described [28]. Prior to testing, aptamers were diluted into a HEPES-based buffers and refolded by heating to 65 °C for 5 minutes followed by cooling for 3 minutes at room temperature.

Recombinant FXIa and FXIa variants

Human FXI was expressed in HEK293 fibroblasts and purified from conditioned media as described [29]. In addition to wild type FXI (FXI-WT), FXI species were prepared with alanine substitutions for (1) Arg²⁵⁰, Lys²⁵², Lys²⁵³ and Lys²⁵⁵ which form an ABS on the FXI A3 domain (FXI-ABS1) [19]; (2) Lys⁵²⁹, Arg⁵³⁰ and Arg⁵³² which form a second ABS on the catalytic domain (FXI-ABS2) [20]; or (3) Arg⁵⁰⁴, Lys⁵⁰⁵, Arg⁵⁰⁷ and Lys⁵⁰⁹ (FXI-504-509) in the catalytic domain autolysis loop [21]. Purified proteins were dialyzed against 25 mM Tris-HCl pH 7.4, 100 mM NaCl (TBS). FXIa was generated by incubating FXI (~300 μ g/ml) in TBS with 5 μ g/ml FXIIa. Complete activation was confirmed by SDS-PAGE. Proteins were stored at -80 °C.

Screening for aptamer activity

Aptamers were screened for their ability to inhibit FXIa cleavage of a chromogenic substrate and FXIa activation of factor IX. FXIa (25 nM) was incubated with refolded aptamer (500

nM) or buffer for 5 min at 37 °C in 20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and 0.01% BSA buffer. The substrate Pefachrome FXIa 3107 (final concentration 1 mM) was added to bring the volume to 100 µL, and changes in OD 405 nm were followed on a kinetic microplate reader. Conversion of factor IX to factor IXa β by FXIa was measured in a two stage chromogenic assay performed at 37 °C in 20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and 0.01% BSA. Aptamer or buffer was incubated with FXIa (final concentration 5 nM) for 5 minutes at 37 °C, and reactions were initiated with the addition of FIX (final concentration 1 µM). At various times, samples of reactions were removed and FXIa activity was quenched with lima bean trypsin inhibitor and EDTA (final concentrations 1 mg/mL and 2.1 mM, respectively). Factor IXa β was measured by the addition of 1 mM Pefachrome FIXa 3107 (Centerchem, Inc., Norwalk, CT) in 20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and 0.01% BSA with 30% ethylene glycol. The rate of substrate hydrolysis was recorded at 405 nm using the microplate reader.

Aptamer binding to FXI or FXIa

A double-filter nitrocellulose binding assay was used to determine the apparent binding affinity constants (K_d values) of aptamer to protein. Bacterial alkaline phosphatase (Gibco BRL, Gaithersburg, MD) was used to dephosphorylate the 5' end of the RNA, which was then end-labeled with [γ -³²P] ATP (Perkin Elmer, Waltham, MA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Various concentrations of purified protein were incubated with [γ -³²P] RNA in 20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and 0.01% BSA at 37°C for 5 minutes. The unbound RNA was separated from the RNA-protein complexes by passing the mixture through a nitrocellulose filter (Protran BA 85, Whatman Inc., Florham Park, NJ). The levels of bound RNA were quantified using a Storm 825 phosphorimager (GE Healthcare, Piscataway, NJ). Non-specific binding of the radiolabeled RNA to the nitrocellulose filter was subtracted, and the data were fitted using nonlinear regression to determine the apparent binding affinity for the RNA aptamer-protein interaction (GraphPad Prism, GraphPad Software, Inc., San Diego, CA).

FXIa cleavage of tripeptide chromogenic substrates S-2366

For kinetic analyses, FXIa-WT (3 nM protein, 6 nM active sites) was incubated with 250 nM aptamer in 50 mM HEPES buffer (50 mM HEPES pH 7.4, 125 mM NaCl, 5 mM CaCl₂) and S-2366 (final concentrations 0 to 1.0 mM). For analysis of FXIa ABS variants, 3 nM FXIa with or without polybrene (0.2 mg/mL final concentration) were incubated with aptamer (1-2000 nM) in 50 mM HEPES buffer containing 500 µM S-2366 (total volume of 100 µL). For all assays, substrate cleavage was followed by monitoring absorbance at 405 nm using a microplate reader. Initial rates of substrate cleavage were determined using linear regression analysis. *p*-nitroaniline generation from tripeptide-*p*-nitroanilide substrates was calculated using an absorption coefficient of 9,933 M⁻¹ cm⁻¹ at 405 nm [30]. K_m and k_{cat} for S-2366 hydrolysis by FXIa-WT and FXIa variants were obtained by initial rate analysis of *p*-nitroaniline generation as a function of S2366 concentration. K_i values for binding of aptamer to FXIa were obtained by simultaneous fitting of substrate and inhibitor dependences of initial rates of S2366 hydrolysis by the general hyperbolic mixed-type inhibition model [31]. Data were analyzed using GraphPad Prism (San Diego, CA) and Scientist software (MicroMath Scientific Software, Salt Lake City, UT) [29].

FIX activation by FXIa

Activation of FIX by FXIa was determined by SDS-PAGE analysis. FIX (200 nM) in 50 mM HEPES pH 7.4 buffer was incubated with aptamers (1000 nM) at 22 ± 1 °C for 10 min. Then FXIa (1.5 nM) was added, and aliquots were removed at various times (0-300 min) into nonreducing SDS-sample buffer. Samples were run on 20% SDS-polyacrylamide gels and stained with GelCode Blue reagent (Pierce). The gels included standards for factors IX and IXa β . Gels were imaged on an Odyssey infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Concentrations of factor IX and FIXa were calculated using densitometry analysis. Estimates for the apparent steady-state kinetic parameters K_m , k_{cat} and k_{cat}/K_m for factor IX activation were obtained by non-linear least-squares fitting of full progress curves for factor IX activation, measured by factor IXa β appearance [32].

Coagulation Assays

Activated partial thromboplastin time (aPTT) assays were carried out using a model ST4 coagulometer (Diagnostica Stago, Parsippany, NJ). Thirty microliters of refolded aptamer diluted in HEPES buffer without BSA (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM CaCl_2) to a concentration of 4.25 μM was added to 30 μL pooled normal human platelet poor plasma and incubated for 2 minutes at 37 °C. 30 μL PTT-A reagent was then added and incubation was continued for 5 minutes. Coagulation was initiated by addition of 30 μL of 25 mM CaCl_2 and the time to clot formation was recorded. Estimated percent inhibition was determined by comparing the aPTT to a standard curve prepared with FXI deficient plasma supplemented with known amounts of human FXI. Comparisons between groups were performed by Mann-Whitney U test.

RESULTS

SELEX and Lead Clone Selection

We used a solution based directed evolution process called systematic evolution of ligands by exponential enrichment (SELEX) to isolate aptamers targeting FXIa. A starting library of $\sim 10^{14}$ 2'-fluoropyrimidine modified RNA sequences, termed "Sel-3", was incubated with FXIa, and high affinity binding sequences were isolated and amplified. As the selection procedure progressed, binding stringency of the selection was increased in later rounds by increasing the ratio of RNA to protein. Because binding of the starting library to FXIa was weak, the initial selection was conducted in low salt buffer (100 mM NaCl) until high affinity binding was achieved. Then, salt concentration was increased to physiologic amounts (150 mM NaCl) for the later selection rounds.

Overall, fourteen SELEX rounds were performed, with progress monitored by assessing the ability of the library at each round to inhibit FXIa cleavage of a tripeptide substrate (Figure 1A). FXIa inhibition was detectable as early as round 4, with maximum potency achieved in rounds 11 and 12. In later rounds FXIa inhibition was reduced slightly as selection stringency was increased to isolate tighter binding aptamers. RNA from rounds 11, 12 and the final round 14 were cloned and sequenced to obtain individual aptamer sequences, which were organized into families. Selected sequences are shown in Table 1 and a more extensive list is provided in Supp. Tables 1 and 2. There was considerable sequence diversity within

each round and between rounds. Early rounds contained many “orphan” sequences that did not fit into a particular aptamer sequence family, and are present at a low frequency in the round pool. Later rounds were dominated by a small number of sequences, with few orphan sequences. Because there was loss of functional sequences in later rounds, we analyzed orphan sequences from rounds 11 and 12 in addition to analyzing aptamer sequence families.

Screening aptamers

The panel of aptamers was tested for their capacity to inhibit FXIa cleavage of a tripeptide substrate (Figure 1B) and FXIa activation of factor IX (Supp. Table 1). At a concentration of 500 nM (20-fold greater than FXIa), aptamers exhibited a range of effects. Several had no effect on either functional assay (results similar to control aptamer). Two of these sequences, Xla7 and Xla8, dominated the round 14 library and were most likely responsible for the reduced inhibitory properties of this pool compared to pools from rounds 11 and 12. We chose the most potent aptamer, 12.7 and an intermediate inhibitor, 11.16, to further characterize. Each aptamer was analyzed using nitrocellulose filter binding assays to determine their binding affinity to both zymogen FXI and FXIa. These studies suggested that 11.16 binds to only FXIa, while 12.7 binds to both zymogen FXI and FXIa (Supp. Table 2). Both aptamers did not exhibit specific binding toward a panel of coagulation factors, including the structurally related prekallikrein (data not shown).

Effects of aptamers 11.16 and 12.7 on FXIa cleavage of S-2366

The impact of aptamer 11.16 and 12.7 binding on FXIa active site function was determined by assessing cleavage of the tripeptide substrate S-2366. 11.16 and 12.7 both inhibited S-2366 cleavage across a range of substrate (Figure 2) and aptamer (Figure 3) concentrations, with patterns largely consistent with non-competitive inhibition. 11.16 and 12.7 caused a ~65 to 85% decrease in k_{cat} and a modest ~1.5-fold increase in K_{m} (Table 2). The K_{i} values for the aptamers were 60 ± 20 and 63 ± 22 nM, respectively (Table 2). Interestingly, the negative control aptamer library Sel-3 was a weak inhibitor of FXIa (K_{i} ~1.5 μM , Figure 3) suggesting a component of aptamer inhibition may be due to a non-specific property such as charge (discussed below).

Effects of aptamers 11.16 and 12.7 on FXIa activation of factor IX

Aptamers were tested for their ability to inhibit FXIa conversion of factor IX to the proteases factor IXa β . As factor IXa β cleaves chromogenic substrates relatively poorly, we used analyses of SDS-PAGE to follow disappearance of zymogen factor IX and appearance of the product factor IXa β (Figure 4 and Supp. Figure 1), as described [29,32]. Aptamers 11.16 and 12.7 significantly delayed factor IX conversion to factor IXa β (Figure 4). Progress curve analysis indicated a decrease of $k_{\text{cat}}/K_{\text{m}}$ from $46 \pm 7 \text{ M}^{-1}\text{s}^{-1}$ to 1.2 ± 0.1 and $1.3 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$ for aptamers 11.6 and 12.7, respectively, again with a pronounced effect on k_{cat} (Table 3). The control aptamer library Sel-3 caused an ~5-fold decrease in $k_{\text{cat}}/K_{\text{m}}$ (Table 3). Similar, modest inhibition of this reaction has been reported for heparin, consistent with a charge-based inhibition [30].

FXIa anion binding sites and aptamer inhibition

FXIa contains positively charged patches (Anion Binding Sites [ABS]) that contribute to interactions with macromolecules involved in FXIa regulation (Figure 5) [19,20,32]. ABS1 on the A3 domain and ABS2 on the catalytic domain are required for interactions with polyanions such as polyphosphate, dextran sulfate, and heparin [19,20]. Basic residues on the catalytic domain autolysis loop are involved in interactions with serine protease inhibitors [21]. As RNA aptamers are negatively charged due to their phosphate-based backbones, we postulated they may interact with one or more of these sites. We tested a panel of FXIa variants in which basic residues within ABS1, ABS2, or the autolysis loop was replaced with alanine (Figure 6, top panel). Previously, we showed that these variants cleave S-2366 and activate factor IX similarly to FXIa-WT [19-21].

Similar to the polyanions such as heparin and polyphosphate, control RNA aptamer had a weak inhibitory effect on S-2366 cleavage by FXIa-WT but not on FXIa variants (Supp. Figure 2). For FXIa-WT, the weak inhibitory effect of Sel-3 control aptamer library (Supp. Figure 2) and the more potent effects of aptamers 11.16 and 12.7 (data not shown) were abolished when the polycation polybrene was added to reactions, consistent with the importance of charge interactions in aptamer binding. 11.16 and 12.7 produced dose-dependent inhibition of substrate cleavage by FXIa-WT (Figure 3). The activity of aptamer 11.16 was abolished by mutations in the FXIa autolysis loop, and partially disrupted by the absence of ABS2 (Figure 6, middle panel, and Table 4). Removing ABS1 had only a modest effect on inhibition. In contrast, the activity of aptamer 12.7 was abolished by the loss of ABS2, while loss of the autolysis loop site partly reduced aptamer function (Figure 6, bottom panel, and Table 4). There was only modest change in 12.7 activity with loss of ABS1.

Effects of aptamers 11.16 and 12.7 in plasma

In a complex environment, such as blood plasma, aptamers 11.16 and 12.7 may interact with proteins other than the intended target, thus affecting performance. FXIa aptamers were tested in plasma for their capacity to inhibit coagulation in a standard aPTT assay, which requires FXI activation by FXIIa, and subsequent factor IX activation by FXIa (Figure 7). The mean aPTT for vehicle treated plasma was 28.4 ± 6.3 seconds. Aptamer 12.7 increased the aPTT to 56.3 ± 14.6 seconds suggesting, on average, inhibition of >90% of the FXI activity in plasma. In contrast, 11.16 increased the aPTT to only 36.4 ± 5.3 seconds, a result similar to that for control aptamer library Sel-3 (40.3 ± 5.0 seconds). This indicates that 11.16 loses potency in plasma, possibly due its decreased affinity towards zymogen FXI as compared to 12.7, which binds to both FXI and FXIa.

DISCUSSION

We used solution based SELEX to isolate aptamers that bind to FXIa. Previous selections using this strategy against coagulation proteins are often driven to near completion, with one or two closely related sequences dominating the pool [28,33]. However, the present selection generated several aptamer families, as well as individual aptamer sequences that did not fit into families. Using functional assays, we identified species with different levels of potency

that inhibited FXIa activity toward a tripeptide substrate and its primary macromolecular substrate factor IX. The ability to inhibit small peptide substrate cleavage indicated that the aptamer inhibitors were binding to the protease catalytic domain. Indeed, the most potent aptamers were isolated from SELEX rounds showing the highest inhibitory activity in chromogenic substrate assays. While aptamers do not usually bind at protease active sites, some, such as our previously described factor IXa inhibitory aptamer, do so [34]. Aptamers are polyanions, and most coagulation proteases, including factor IXa β and FXIa (Figure 5), have ABSs on catalytic domain surface loops that are in proximity to the protease active site [20,35]. It is hypothesized that aptamers bind preferentially at or near these positively charged patches. Aptamers 11.16 and 12.7 both engage FXIa ABS2 and a charged area on the FXIa autolysis loop. As shown in Figure 5, a large molecule spanning these regions would probably interfere with access to the active site. So, while allosteric changes may occur upon aptamer binding, steric interference with substrates likely occurs as well. This hypothesis is consistent with the non-competitive patterns of inhibition seen for both S-2366 and factor IX cleavage. Recently described synthetic heparin-mimetic compounds that inhibit FXIa also bind in the vicinity of ABS2 and may work by a similar combined mechanism as the aptamers [36,37].

Somewhat surprisingly, we did not identify aptamers that inhibited factor IX activation without affecting tripeptide substrate cleavage. Previously, an anti-FXIa antibody (O1A6) that binds in proximity to ABS1 was shown to be a potent FXIa inhibitor in clotting assays because it interferes with an exosite on the A3 domain that is critical for factor IX binding [11,29]. The antibody did not affect tripeptide substrate cleavage by FXIa. In the present study, we did not identify aptamers that bind near ABS1 that produced similar effects to O1A6. With the advent of deep sequencing techniques, the aptamer pool can be probed to identify sequences that might have properties similar to A3 domain-directed antibodies.

The anionic polymers polyphosphate and heparin produce different effects when they bind to FXIa [19,20,38]. Polyphosphate binding requires both ABS1 and ABS2 [38], and a single polymer could potentially bind to both sites simultaneously. Polyphosphates accelerate FXI activation by thrombin and FXIIa and have a procoagulant effect in blood [18,38]. Heparin also uses ABS1 and ABS2 to bind to FXIa, but produces an inhibitory effect in plasma by accelerating antithrombin inhibition of FXIa [19,20]. Hypothetically, drugs blocking the FXIa ABSs could have procoagulant or anticoagulant effects. Previously, we observed that loss of ABS2 reduces FXIa activity in an arterial thrombosis model in mice, suggesting that ABS blockade would produce, on balance, an antithrombotic effect [38].

FXI and its homolog prekallikrein, along with FXII and the cofactor high-molecular weight kininogen, are referred to collectively as contact factors [6-8]. While deficiency of any one contact factor produces an antithrombotic effect in mice [4,5,9,39], data from human populations suggests FXI is the greater contributor to common conditions such as venous thromboembolism and ischemic stroke in humans [3]. In a recent study, antisense oligonucleotide-mediated down regulation of FXI production successfully prevented venous thrombosis during knee replacement therapy [13]. This approach is not suitable for situations requiring rapid anticoagulation, as it takes several weeks for the treatment to take effect. Development of agents such as aptamer inhibitors that can rapidly inactivate FXI/

FXIa are required to fully assess the potential of targeting this protease as an antithrombotic strategy. As synthetic molecules, aptamers have a small molecular weight (8-15kDa), are rapidly cleared by the renal system, and are highly amenable to modifications that can control their bioavailability, allowing for rapid and stable anticoagulation upon administration (within the order of minutes) that can last from hours to days, depending on the specific formulation of the aptamer [24]. Importantly, aptamers have the ability to address therapy-induced bleeding by neutralizing the inhibitor aptamer with a suitable antidote aptamer, thus leading to rapid reversal or control of the level of anticoagulation and increasing the safety of this class of drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Factor XIa contributes to thrombotic disorders and is a target of therapeutic interest.
- A directed evolution approach was used to isolate RNA aptamers targeting Factor XIa.
- Two aptamers bind to anion binding and serpin binding sites on the Factor XIa catalytic domain.
- These aptamers act as noncompetitive inhibitors of Factor XIa.

Figure 1. Anti-FXIa activity of SELEX products

Shown are (A) inhibitory activity in aptamer pools from specific SELEX rounds and (B) inhibitory activity of specific aptamer species. FXIa (25nM) was incubated with SELEX RNA (500 nM), and residual FXIa active site activity was measured using a FXIa specific chromogenic substrate. The data were normalized to the rate of substrate cleavage in the absence of RNA (Buffer – arbitrarily assigned a value of 1.00). Shown are means $\pm$ SEM of duplicate measures.

Figure 2. Inhibition of FXIa by aptamers as a function of chromogenic substrate concentration FXIa (3 nM) was incubated with buffer (●) or 250 nM aptamer (○) and various concentrations of the chromogenic substrate S-2366. Linear initial rates of generation of *p*-nitroaniline were measured by continuous monitoring of absorbance at 405 nm, and graphed for each substrate concentration.

Figure 3. Inhibition of FXIa cleavage of chromogenic substrate as a function of aptamer concentration

FXIa (3 nM) was incubated with varying concentrations of control aptamer library Sel-3 (●), 11.16 (○), or 12.7 (gray circle). Residual FXIa activity was determined using the chromogenic substrate S-2366. Linear initial rates of generation of *p*-nitroaniline were measured by continuous monitoring of absorbance at 405 nm, and graphed for each aptamer concentration.

Figure 4. Aptamer inhibition of FXIa activation of factor IX

Time courses of activation of factor IX (200 nM) by FXIa (1.5 nM) in the presence of 1000 nM Sel-3, 11.16, or 12.7 RNA aptamers. Conversion of factor IX to factor IXaβ was determined by densitometry of Coomassie stained SDS-polyacrylamide gels. Loss of zymogen factor IX (●, ■) and appearance of factor IXaβ (○, □) are plotted in the absence (○, ●) and presence (□, ■) of aptamer. The lower right hand panel shows formation of factor IXaβ over time in the absence of aptamer (○) or in the presence of 1000 nM Sel-3 (●), 11.16 (□), or 12.7 (■). Data is a representative example of two independent experiments.

Figure 5. FXI Anion Binding Sites (ABSs)

Space-filling models of FXI (left) and the FXI catalytic domain (right) [15,40]. The positions of apple domains 2, 3, and 4 (A2, A3 and A4) are shown. With the A1 domain (not seen in this image), they form a platform on which the catalytic domain (CD) rests. Basic residues in ABS1 (green) on the A3 domain; and ABS2 (red) and the autolysis loop (blue) on the catalytic domain are shown. Numbers refer to amino acids within the mature FXI subunit [40]. The position of the active site residue Ser⁵⁵⁷ is shown in orange. Basic residues in the autolysis loop are required for aptamer 11.16 inhibitory activity, with some contribution from ABS2. Aptamer 12.7, in contrast, requires ABS2, with some contribution from the autolysis loop.

Figure 6. Importance of FXIa anion binding sites (ABSs) for aptamer inhibition of FXIa (Top Panel) Coomassie-stained SDS-polyacrylamide gel showing recombinant FXIa species run under reducing conditions. (Middle and Bottom Panels) FXIa-WT (○), FXIa-ABS1 (gray circle), FXIa-ABS2 (●) and factor XIa-Ala-504-509 (□), at 3 nM concentration, were incubated with varying concentrations of aptamer 11.16 (Top) or 12.7 (Bottom). Residual FXIa activity was measured using the chromogenic substrate S-2366 (500 nM). Initial rates of generation of *p*-nitroaniline were measured by continuous monitoring of absorbance at

405 nm, and graphed for each aptamer concentration. The data represent the means of duplicate measures.

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Figure 7. FXIa aptamers in activated partial thromboplastin time assays

Each symbol represents an individual aPTT for normal plasma in the absence of aptamer (●) or in the presence of 4.25 μ M aptamer 12.7 (■) or 11.16 (▲). Large horizontal bars indicate means for the group. * $p < 0.02$ for each aptamer group compared to control; ** $p = 0.001$ for 12.7 compared to Sel-3.

Table 1
Frequency of selected RNA aptamer sequences in indicated rounds from FXIa solution-based SELEX

SELEX rounds 11, 12 and 14 were cloned and sequenced to determine individual aptamer sequence frequency in each round. For additional sequences and full DNA template and RNA sequences, refer to Supplemental Tables 1 and 2.

Aptamer	Random Region Sequence	Round Frequency (%)		
		R11	R12	R14
Xla1	ACCGCAUCCGUGAAGAUGCCUCUUAUCCCCUCCCCC	17.4	17.4	20.8
Xla2	AAUUACCCGCGUCUGUAGUACACAUGCUAUCCCCUCCCCC	17.4	0	8.3
Xla4	AUCGUGCAUUAUUUCUGGCUACCAGCCAACGUGCCCCCCC	4.3	13.0	0
Xla7	CAAACUAAUUCGGGCCGCGCACUGGGUCCUCCCCCCC	0	0	25.0
Xla8	GCCGUCCUGAGUCGUAUGAAUCCGCAUGGUCGUCGUGUG	0	8.7	29.2
11.16	CCAGUCCGCAUCCAUAUCCCCUCCCCC	4.3	0	0
12.7	UAACGCCACGCUCGACAACGCGUCGAGUGUCCUCCGCCCC	0	4.3	0

Table 2
Effect of RNA aptamers on kinetic parameters for FXIa cleavage of S-2366

Parameters were obtained by simultaneous fitting of the S-2366 and aptamer dependences. K_m and k_{cat} are the apparent values, obtained by multiplying the fitted K_m and k_{cat} values for each data set in the absence of aptamer by α and β , respectively.

	No Aptamer	Sel-3 Control	11.16	12.7
K_m (μM)*	290 ± 20	450 ± 120	440 ± 60	410 ± 70
k_{cat} (sec ⁻¹)*	31 ± 2	18 ± 2	4.6 ± 0.3	10.6 ± 0.6
K_i (μM)	-	2 ± 3	0.060 ± 0.020	0.063 ± 0.022
α (K_m factor)	1	2 ± 6	1.6 ± 1.0	1.5 ± 0.8
β (k_{cat} factor)	1	0.6 ± 1.0	0.14 ± 0.05	0.32 ± 0.08

Table 3
Effect of RNA aptamers on kinetic parameters for FXIa activation of factor IX

Best fits for k_{cat} were obtained by simplex fitting, and subsequently fixed for nonlinear least squares analysis of K_{m} , using the integrated Michaelis-Menten equation. $k_{\text{cat}}/K_{\text{m}}$ values were calculated from the fits.

	No Aptamer	Sel-3 Control	11.16	12.7
$K_{\text{m}}(\mu\text{M})^*$	0.26 ± 0.04	0.60 ± 0.09	3.1 ± 0.1	2.9 ± 0.4
$k_{\text{cat}} (\text{min}^{-1})^*$	12	5.4	3.7	3.7
$k_{\text{cat}} / K_{\text{m}} (\mu\text{M}^{-1}\text{min}^{-1})$	46	9	1.2	1.3

Table 4
Effects of RNA aptamers on kinetic parameters for cleavage of S-2366 by FXIa variants

Analysis of the aptamer dependences was performed using K_m and k_{cat} values determined independently by simplex fitting in the absence of aptamer, and fixing the best fits in nonlinear least squares analysis of K_i , α and β . Apparent K_m and k_{cat} values in the presence of aptamer were obtained by multiplying these fixed K_m and k_{cat} values by the fitted α and β , respectively.

	No Aptamer	Sel-3 Control	11.16	12.7
FXIa-ABS1				
K_m (μM)*	311	-	430 ± 120	500 ± 300
k_{cat} (s^{-1})*	36	-	7 ± 1	9 ± 3
K_i (μM)	-	-	0.08 ± 0.01	0.28 ± 0.07
α (K_m factor)	1	-	1.4 ± 0.4	1.6 ± 0.9
β (k_{cat} factor)	1	-	0.19 ± 0.03	0.26 ± 0.07
FXIa-ABS2				
K_m (μM)*	285	-	510 ± 20	-
k_{cat} (s^{-1})*	35	-	12 ± 2	-
K_i (μM)	-	-	0.25 ± 0.04	-
α (K_m factor)	1	-	1.8 ± 0.6	-
β (k_{cat} factor)	1	-	0.33 ± 0.06	-
FXIa- Aut Loop				-
K_m (μM)*	290	-	-	460 ± 60
k_{cat} (s^{-1})*	36	-	-	26 ± 18
K_i (μM)	-	-	-	0.28 ± 0.08
α (K_m factor)	1	-	-	1.6 ± 0.2
β (k_{cat} factor)	1	-	-	0.72 ± 0.05