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## Modulation of the coagulation cascade using aptamers

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### Abstract

As a novel class of therapeutics, aptamers, or nucleic acid ligands, have garnered clinical interest due to the ease of isolating a highly specific aptamer against a wide range of targets, their chemical flexibility and synthesis, as well as their inherent ability to have their inhibitory ability reversed. The following review details the development and molecular mechanisms of aptamers targeting specific proteases in the coagulation cascade. The ability of these anticoagulant aptamers to bind to and inhibit exosite function rather than binding within the active site highlights the importance of exosites in blocking protein function. As both exosite inhibitors and reversible agents, the use of aptamers is a promising strategy for future therapeutics.

### Keywords

aptamer; anticoagulation; coagulation cascade

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Aptamers are small, single-stranded oligonucleotides whose linear sequences encode specific three-dimensional structures that allow them to bind to their target proteins or other molecules with high affinity and specificity. By screening large libraries of single stranded oligonucleotides in a combinatorial chemistry process termed SELEX, or systematic evolution of ligands by exponential enrichment, scientists can identify the oligonucleotide sequence or sequences responsible for binding to various molecules with high affinity<sup>1,2,3</sup>. This technology has been exploited to isolate aptamer sequences to a growing number of proteins, including growth factors, transcription factors, viral proteins and coagulation factors, a number of which are currently in clinical trials and which have been reviewed extensively elsewhere<sup>4–6</sup>.

Crystal structures of aptamers bound to proteins have shown that aptamers fold into a tertiary structure and present an extended conformational surface that is complementary to the surface of its target, thus binding and burying a large surface area ( $\sim 1,000\text{\AA}^2$ ) on the

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protein<sup>7, 8</sup>. Because of the numerous specific interactions along the extended binding interface, aptamers often bind their targets with high affinity (dissociation constants in the low nanomolar range) and specificity. Additionally, since a large part of the target protein is concealed by aptamer binding, aptamers tend to act as antagonists by blocking protein-protein interactions rather than inhibiting active site activity.

A unique benefit of aptamers as anticoagulants is their ability to be therapeutically regulated, either by controlling their circulating half-life or reversing their function with an antidote. Unmodified RNA aptamers are rapidly degraded in biological fluids by endogenous endonucleases, leading to extremely short half-lives. While DNA aptamers are inherently more stable, RNA is thought to be able to adopt more complex conformations which may allow for different binding patterns and higher affinity to a target protein. To increase stability of the RNA aptamers, chemically modified nucleotides are incorporated in the aptamer sequence during the selection process in order to prevent endonuclease cleavage<sup>9</sup>. Highly modified RNA aptamers are even more resistant to nuclease degradation than DNA aptamers in human blood. In addition, other strategies are used to further increase aptamer stability, such as capping the ends of the aptamer to protect it from exonuclease cleavage<sup>10</sup>. Both DNA aptamers and modified RNA aptamers are relatively small (8–15 kDa) and are therefore rapidly cleared by the renal system<sup>11</sup>. To further increase the circulating half-life of an aptamer, bulky, inert moieties, such polyethylene glycol (PEG) or lipophilic moieties such as cholesterol, can be conjugated to the 5' end of an aptamer to increase the molecular weight and thereby decrease the renal clearance rate. In this way, the bioavailability of an aptamer can be manipulated depending on its eventual use, with cholesterol conjugation of an aptamer typically increasing the half-life to several hours<sup>12</sup> and PEG conjugation extending the half-life to days or even weeks, depending on the mode of administration<sup>6, 13</sup>.

While the use of an unmodified or rapidly cleared aptamer would require a large amount of compound to produce an effect over a given amount of time, antidote administration to control a long-circulating aptamer formulation allows for the use of lower amounts of active compound. Two types of antidotes have been developed that can rapidly reverse the activity of the aptamer while leaving the target protein intact and functionally available. An oligonucleotide-based antidote recognizes and binds to the primary sequence of the aptamer by Watson-Crick base pairing, resulting in a disrupted aptamer structure that can no longer bind to its target protein<sup>14</sup>. A universal antidote can bind to the backbone of any aptamer in a sequence independent manner and thereby sequester any aptamer and subsequently reverse its effects<sup>14, 15</sup>. Both types of antidotes would allow clinicians to respond immediately to an urgent clinical need by titrating in the amount of antidote needed. For anticoagulation, this aptamer-antidote system has received significant attention for its ability to control anticoagulation, and therefore bleeding, to the degree required.

Current anticoagulation strategies have been hampered by nonselective agents, agents with narrow therapeutic windows, and agents with no dosing flexibility or the ability to be reversed. The application of the aptamer platform to anticoagulation has the ability to fill this void. Aptamers targeting several members of the coagulation pathway have been developed and progressed into clinical trials, and the first aptamer-antidote system, targeting factor IXa (FIXa), advanced to phase 3 clinical trials. Overall, this system exhibited a rapid

and reproducible onset of anticoagulation in a readily measurable manner, with the ability to be predictably controlled with antidote administration<sup>16, 17</sup>. While this aptamer-antidote system has paved the way for the translation of other aptamer-based antithrombotics, this review will detail the development of several aptamer-based inhibitors against members of the coagulation cascade (Table 1 and Figure 1).

## Prothrombin/Thrombin Aptamers

Thrombin is the final step in the blood coagulation pathway, and mediates many important features of blood coagulation, including cleaving fibrinogen into fibrin, activating platelets, and amplifying the coagulation cascade<sup>18</sup>. Thrombin has three functionally important binding sites: the catalytic active site, and two anionic binding sites, exosite I and II, which are located on opposite sides of the protein. Pro-exosite I is present on both prothrombin and thrombin, and binds to fibrinogen, factor V (FV), factor VIII (FVIII), thrombomodulin, and platelet PAR receptors<sup>18</sup>. In this way, pro-exosite I mediates fibrinogen cleavage, feedback activation of clotting cofactors, and platelet activation. The other anion binding site, exosite II, is only present on thrombin and binds to heparin, FV, FVIII, and the platelet receptor GPIIb<sup>18</sup>. Therefore, exosite II also mediates platelet activation, as well as inhibition by heparin. Thus, inhibitors targeting different sites on thrombin have different modes of action, and combining inhibitors to both exosites can result in a very potent inhibition of thrombin function<sup>19</sup>.

Because of thrombin's central role in coagulation, this well-characterized protein has been the target for some of the first *in vitro* aptamer selections. The first aptamer to be isolated to thrombin was also the first aptamer isolated to a non-nucleic acid binding protein. This aptamer, termed HD1 (also known as ARC183) is an unmodified 15 nucleotide single-stranded DNA aptamer that forms a stable G-quadruplex and binds to pro-exosite I on both prothrombin and thrombin<sup>20-22</sup>. This aptamer inhibits pro-exosite I functions, including fibrinogen and FV cleavage, and competes with FVa for prothrombin binding, thus inhibiting prothrombinase function<sup>20, 23</sup>. In addition, HD1 blocks platelet PAR interactions with pro-exosite I to inhibit thrombin mediated platelet activation and aggregation<sup>24</sup>. Unlike heparin, which inhibited only 35% of clot bound thrombin at clinically relevant doses, HD1 inhibited more than 80% of clot-bound thrombin in *ex vivo* studies<sup>25</sup>. Due to its anticoagulant activity in human plasma, this aptamer was explored in several animal models as a cardiopulmonary bypass agent. In cynomolgous monkeys, HD1 has a very short half-life (108 seconds) and rapid clearance by the body (2-4 minutes); therefore, a continuous infusion was needed to achieve efficacious anticoagulation as monitored by an increase in a prothrombin time assay<sup>26</sup>. Once administration was halted, coagulation values returned to baseline in approximately 10 minutes, abolishing the need for a reversal antidote. Similar studies in canines determined that the aptamer compound was well tolerated, and exhibited similar anticoagulant profiles as in monkeys with continual infusion of compound<sup>27</sup>. While preliminary human studies were commenced, the large quantities of aptamer required for continual administration for anticoagulation resulted in a suboptimal dosing profile, and the human clinical trials were ended<sup>28</sup>. Moreover, the variation in renal function of patients undergoing cardiopulmonary bypass makes dosing and monitoring of such rapidly clearing anticoagulant agents challenging.

Another DNA aptamer, Nu172, was developed by Archemix/Nuvelo to be a more potent, short-acting thrombin aptamer than ARC183. Modeled after ARC183, this 26-nucleotide aptamer produced a dose dependent increase of clinical coagulation assays during phase I testing in healthy male volunteers. Since this aptamer has a short half-life, after terminating infusion of Nu172, coagulation values quickly returned to baseline without requiring the use of an antidote<sup>29</sup>.

HD22 is another thrombin-binding unmodified DNA aptamer that is 29 nucleotides long and also forms a G-quadruplex. This aptamer, however, binds to exosite II on thrombin to inhibit thrombin-mediated activation of platelets and FV/FVIII activation, but has minimal effects on fibrinogen cleavage<sup>30</sup>. A very potent DNA aptamer was made by designing a hybrid aptamer joining both HD1 and HD22 with a poly-dA linker. HD1–22 thus binds to both exosites and exhibits tighter binding and more efficacious anticoagulation than either of the two aptamers alone<sup>31</sup>.

While many of the first thrombin aptamers were DNA, several RNA aptamers targeting thrombin have since been developed. Whereas unmodified DNA has a very short half-life in the body, RNA can be modified to be resistant to endonuclease cleavage and greatly increase an aptamer's plasma stability. A 25 nucleotide 2' fluoropyrimidine RNA aptamer, Tog25, was selected against thrombin using a "toggle" SELEX method where the selection was toggled back and forth each round with human and porcine thrombin in order to promote species cross-reactivity<sup>32</sup>. Tog25 binds to exosite II of thrombin, thus inhibiting thrombin-mediated platelet activation but having a minimal effect on fibrinogen cleavage<sup>33</sup>. In contrast to the G-quadruplex architecture of the DNA aptamers, Tog25 has a traditional stem-loop structure with an internal bulge. A crystal structure of Tog25 in complex with human thrombin shows that the RNA forms an elaborate three-dimensional structure to present an extended molecular surface complementary to the protein. A number of key interactions, including an "A-Arg zipper", which involves a number of adenine-arginine stacking interactions, as well as hydrogen bonds and van der Waals interactions, contribute to the intricate folding that allows Tog25 to tightly interact with thrombin<sup>7</sup>. While Tog25 is not a potent anticoagulant, dual administration with the pro-exosite I binding DNA aptamer, HD-1, results in synergistic anticoagulation, similar to the bivalent DNA aptamer described above<sup>19</sup>.

Recently, another 2' fluoropyrimidine modified RNA aptamer that binds to thrombin was described<sup>34</sup>. R9d14t is 58 nucleotides and binds to both prothrombin and thrombin at pro-exosite I, thus inhibiting fibrin clot formation, FV feedback activity, and platelet activation mediated by the PAR receptors. In addition, because the aptamer binds to pro-exosite I on prothrombin, it inhibits thrombin generation by the prothrombinase complex of FXa/FVa. This aptamer is a dose-dependent, potent inhibitor of coagulation in clinical clotting assays and an oligonucleotide antidote was developed to rapidly and stably reverse this anticoagulation. While this aptamer binds to the same exosite as the DNA aptamer HD-1, R9d14t is a more potent inhibitor of thrombin due to its increased stability and higher binding affinity<sup>34</sup>.

## Factor Xa (FXa) Aptamer

FXa combines with its cofactor, FVa, on the surface of a platelet to cleave prothrombin to thrombin. While FXa has some protease activity itself, the formation of the prothrombinase complex yields a marked increase in thrombin generation. Since prothrombinase cleavage of prothrombin is the only reaction not duplicated in the coagulation pathway, inhibition of this complex would yield a potent anticoagulant. Several new oral anticoagulants specifically targeting FXa have recently shown remarkable efficacy in preventing stroke and managing venous thromboembolism, although none of these currently have an effective antidote approved for use<sup>35</sup>. An aptamer, 11F7t, was isolated that binds tightly to FX and FXa with an apparent dissociation constant of approximately 1nM<sup>36</sup>. This aptamer works as a potent anticoagulant in clinical coagulation assays by blocking the assembly of the prothrombinase complex. Biochemically, 11F7t binds to FXa and competes for binding with FVa. Therefore, in the presence of aptamer, the prothrombinase complex cannot form, resulting in a decreased amount of thrombin able to be produced (Figure 1B). Albeit its potent anticoagulant effect, the aptamer does not affect the ability of FX to bind to a phospholipid membrane surface, its catalytic site activity, or influence substrate binding. In addition, the aptamer 11F7t blocks the ability of FX to be activated by the FVIIIa/FIXa complex, but does not have an effect on FX activation by the FVIIa/TF complex. On the other hand, the aptamer blocks tissue factor pathway inhibitor (TFPI) from inhibiting FXa. Thus, the aptamer biochemically exhibits both procoagulant and anticoagulant effects, and this emphasizes the importance of the net effect of the interactions with which the aptamer interferes to exhibit its anticoagulant effect<sup>36</sup>.

## Factor VII (FVIIa) Aptamer

The principal activator of the extrinsic pathway, and also the major activator *in vivo*, is the TF/FVIIa complex. Upon TF exposure, FVIIa binds to TF, allowing FVIIa to then cleave FIX and FX to their active proteases, and thus initiate thrombin generation at the site of injury. An inhibitor against FVIIa would therefore shut down the initiation of thrombin generation. A 2' aminopyrimidine RNA aptamer, termed 16.3, was generated that binds to FVII/FVIIa with a disassociation constant of 10nM. This aptamer inhibits the TF/FVIIa mediated activation of FX, at least in part by preventing this complex to form<sup>37</sup>. In a prothrombin time assay, this aptamer could dose dependently prolong the clotting time. Unfortunately, the folding of aptamer 16.3 is temperature sensitive, and at 37°C, has decreased binding affinity for FVIIa. Therefore, this aptamer was not further developed clinically. Subsequently, FVII binding aptamers were isolated using a convergent SELEX approach, where five rounds of selection against the GLA proteome was performed followed by two rounds of selection against purified FVII. Two 2' fluoropyrimidine RNA aptamers were identified that bound to FVII at 37°C with similar disassociation constants of ~60nM, but differed in their anticoagulant potency in a prothrombin time assay<sup>38</sup>.

## Factor IXa (FIXa) Aptamer

Although a member of the intrinsic pathway, FIX can be activated by both FXIa and the TF/FVIIa complex and is thus involved in both pathways. FIXa then forms a complex with its

cofactor, FVIIIa, to activate a large amount of FX on the platelet surface, leading to stable fibrin clot formation. A 35 nucleotide, 2' fluoropyrimidine modified RNA aptamer termed 9.3t was isolated that binds to both FIX and FIXa. Biochemical studies indicate that 9.3t blocks an extended substrate binding site so as to inhibit FIX-mediated activation of FX, but it does not inhibit FVIIIa/FIXa complex formation (Figure 1C)<sup>39</sup>. Saturating doses of this aptamer can inhibit greater than 99% of FIXa activity and results in prolonged in vitro plasma clotting times. In addition, a number of short 2'-O-methyl modified RNA antidote oligonucleotides complementary to different portions of the aptamer sequence were identified that could bind to the FIXa aptamer and rapidly reverse all aptamer activity<sup>14</sup>. The most potent of these antidotes was able to reverse anticoagulation within ten minutes in human plasma, and this effect was stable over five hours<sup>14</sup>.

The FIXa binding aptamer was also effective in several animal models. In a murine ferric chloride injury model, a cholesterol modified version of 9.3t protected mice from occlusive thrombus formation<sup>12</sup>. Additionally, administration of the aptamer-antidote pair in swine exhibited reproducible anticoagulation and subsequent reversal, with the antidote neutralizing greater than 95% of anticoagulant effects within 10 minutes<sup>12</sup>. Use of the aptamer-antidote system in a one-hour, neonatal porcine cardiopulmonary bypass surgery model was as effective as using heparin and protamine for reversible anticoagulation<sup>40</sup>. Overall, these preclinical studies established the FIXa aptamer-antidote pair to be a predictable and nontoxic reversible anticoagulation system, and the two compounds (aptamer and antidote) were optimized for clinical studies as the REG1 Anticoagulation System (Regado Biosciences, Durham, NC)<sup>29</sup>.

Phase I clinical studies established REG1 as a safe, effective, and reproducible anticoagulation system in healthy patients or patients with stable coronary artery disease on antiplatelet therapy (Table 2)<sup>13, 16, 41</sup>. Bolus or weight-adjusted administration of RB006, the optimized version of the FIXa aptamer 9.3t conjugated to a 40kDa PEG carrier, resulted in a dose-dependent increase in clinical clotting assays over a 24 hour period<sup>13</sup>. Subsequent administration of RB007, the 2' OMe modified 15-nucleotide RNA antidote, led to reversal of anticoagulant activity within one to two minutes in both patient populations<sup>16</sup>. No rebound anticoagulation was seen over 72 hours, indicating that the aptamer/antidote complex was stable with irreversible binding of aptamer to antidote. While clearance of the free RB006 aptamer involves mainly intravascular and some renal mechanisms, clearance of the antidote RB007, which is not formulated with a PEG, is achieved by rapid renal filtration, allowing for the re-administration of aptamer with immediate restoration of anticoagulant effects<sup>16, 29</sup>. Across these phase I studies, no major bleeding events or serious adverse side effects were seen with REG1 use. Importantly, drug reversal could be closely controlled by titrating in antidote as needed to either fully or partially neutralize the anticoagulant effects of the aptamer to the necessary degree intended<sup>41</sup>. These initial studies were highly significant because they were the first reported clinical studies on any aptamer administered systemically.

Because of the success of the phase I studies, REG1 was next compared to unfractionated heparin as the anticoagulant for elective percutaneous coronary intervention (PCI) in patients with stable coronary artery disease in the phase 2a pilot study Reversal-PCI (NCT00715455)



(Table 2)<sup>42</sup>. Patients received the antiplatelet drugs aspirin and clopidogrel, as well as an appropriate dose of the aptamer RB006 to inhibit greater than 99% of FIXa activity. Anticoagulation could be predictably controlled with either full or partial aptamer reversal with the antidote RB007, and overall the REG1 system exhibited low patient-to-patient variability with stable anticoagulation and reversal<sup>17</sup>. A larger phase 2B study termed RADAR (A Randomized, Partially Blinded, Multicenter, Active-Controlled, Dose-Ranging Study Assessing the Safety, Efficacy, and Pharmacodynamics of the REG1 Anticoagulation System in Patients with acute coronary syndrome) (NCT00932100) was designed to assess the degree of reversal required to prevent thrombosis and control bleeding in planned cardiac catheterization<sup>43</sup>. A total of 640 patients with acute coronary syndrome were randomly assigned to one of four REG1 treatment arms, all receiving a weight adjusted dose of the FIX aptamer RB006 (now termed pegnivacogin) with four increasing levels of antidote RB007 (now termed anivamersen) to achieve 25%–100% reversal<sup>44</sup>. Overall, the REG1 system effectively anticoagulated patients undergoing invasive cardiac procedures while preventing thrombotic and bleeding events in patients receiving at least 50% reversal. Moreover, a trend toward reduced ischemic events and bleeding was observed in patients receiving REG1<sup>44</sup>. While the incidence of adverse events was similar with REG1 and heparin, enrollment in RADAR was halted after three allergic events, albeit with sufficient clinical data from a total of 640 patients enrolled<sup>44</sup>. Despite the three adverse events, a phase 3, 13,000 patient clinical trial, REGULATE-PCI, was initiated to test the REG1 system compared in PCI compared to bivalirudin (A Study to Determine the Efficacy and Safety of REG1 Compared to Bivalirudin in Patients Undergoing PCI) (NCT01848106). However, the trial was terminated early after enrolling 3,232 patients due to a recommendation from the trial's data and safety monitoring board, purportedly concerning rare, severe allergic reactions, although data from the trial has yet to be published (Regado Biosciences, Inc., now Tobira Therapeutics). It has not been reported to date the frequency or what elicited such allergic reactions but since the trial passed through its 1,000 patient safety assessment it is likely that the events are rare. Moreover, since the event occurred prior to antidote administration, it has been speculated that the allergic reactions must be due to recognition of either the aptamer or PEG portion (the high molecular weight modification that was appended to the end of the aptamer to increase circulating half-life) of pegnivacogin. To this end, no immune reactions or complement activation due to any aptamer administration have been reported in pre-clinical or clinical trials. However, antibodies to PEG have been detected both in animal studies<sup>45, 46</sup> and in humans<sup>47, 48</sup>. In addition, there is some evidence that PEGylated compounds can induce complement activation leading to hypersensitivity reactions and anaphylactic shock, although the mechanism involved is not determined<sup>49, 50</sup>. It is not clear if a response to PEG or pre-existing PEG antibodies could have caused the adverse reactions seen in the clinical trials, and we await the publication of the REGULATE-PCI data to shed light on this question.

Overall, REG1 is the first aptamer-antidote pair to be tested in humans, and aside from rare, adverse reactions in later clinical trials, this system has been shown to be a successful anticoagulant strategy with extreme and rapid control over drug activity in many hundreds of patients. While the REG1 system was administered intravenously in these studies, a phase I clinical trial of a subcutaneous administration of the system (termed REG2) was able to

increase the duration of anticoagulation to a half-life of approximately six days (Table 2)<sup>51</sup>. Subsequent antidote administration successfully reversed anticoagulation, but did not inhibit further absorption of aptamer into the bloodstream. No adverse reactions were reported during the trial<sup>51</sup>. As the first subcutaneously administered aptamer, it is unclear whether this mode of administration will affect the adverse reactions seen during intravenous application in the REGULATE-PCI trial, but could offer an additional strategy for long-term anticoagulation.

## Factor XII (FXII) Aptamer

FXII is the initiating member of the contact pathway, and is activated when exposed to a negatively charged surface, including substances, such as collagen, extracellular nucleic acids, or polyphosphates, that are present in the bloodstream at the site of a growing thrombus. Once activated, FXIIa then cleaves FXI to initiate the coagulation cascade, leading to thrombin generation and fibrin clot formation. FXIIa also activates kallikrein, leading to bradykinin-induced proinflammatory signaling. Activation of the contact pathway has been implicated in thrombus formation either on the surface of a foreign material, such as a catheter, or during inflammatory events, such as plaque rupture in atherosclerosis<sup>52, 53</sup>. However, members of the contact pathway are not considered essential in hemostasis, as patients deficient in FXII or kallikrein do not present with a bleeding phenotype, and some patients deficient in FXI may have a mild hemophilia. Inhibition or deficiency of FXII has been effective at limiting thrombosis without interrupting hemostasis in several animal models.

Recently, a 2' fluoropyrimidine modified RNA aptamer inhibitor of FXII has been isolated<sup>54</sup>. This aptamer, R4cXII-1, binds to both FXII and FXIIa, but has no effect on active site activity. While the aptamer itself does not induce the activation of FXII, it does block the ability of other negatively charged reagents, such as kaolin or ellagic acid, from activating FXII. In addition, R4cXII-1 blocks the ability of FXII to activate FXI to decrease thrombin generation and thus increase the clotting time in plasma-based assays activated through the intrinsic pathway. This aptamer, however, is specific for inhibition of the coagulation cascade, as it does not block the ability of FXIIa to activate kallikrein<sup>54</sup>.

## Other aptamers targeting coagulation proteins

While this review focuses on aptamers targeting the proteases of the coagulation cascade, several aptamers have been identified against other proteins involved in other aspects of coagulation, such as anticoagulant proteins, platelet receptors, and von willebrand factor (VWF), that will be briefly mentioned. A panel of single stranded DNA aptamers was isolated to the anticoagulant protein activated protein C, and these aptamers bind to the main exosite on APC to inhibit cleavage of FVa and FVIIa and thus inhibit anticoagulant function<sup>55</sup>. In addition, an aptamer targeting TFPI was developed to treat hemophilia, and this aptamer, ARC19499, interacts with multiple domains on the protein to inhibit its function both *in vitro* and *in vivo*<sup>56-61</sup>. Because of its efficacy as a procoagulant, this aptamer was tested in a Phase I clinical trial in hemophilia patients (Table 2). However, the study was prematurely terminated due to increased bleeding events. Mechanistically, this



side effect was reported to be due to several reasons, namely that aptamer binding decreased TFPI clearance and thus increased its circulatory half-life, leading to an actual increase in the amount of TFPI, resulting in the increased anticoagulant activity seen in patients<sup>62</sup>. Aptamers have also been developed as antiplatelet agents, with an aptamer-antidote system preliminarily described against the collagen platelet receptor glycoprotein VI<sup>63</sup>. Additionally, several aptamers targeting VWF have been developed<sup>64, 65</sup>, and the aptamer ARC1779 advanced in clinical trials for acute coronary syndromes, von Willebrand disease (VWD), and thrombotic thrombocytopenic purpura, among other indications (Table 2)<sup>66–68</sup>. The development, mechanisms, and clinical trials of the VWF aptamer have been reviewed extensively elsewhere<sup>6, 29, 69, 70</sup>, and we refer the reader to the cited literature for additional discussion on these aptamers.

## Conclusions

As shown in Table 1 and Figure 1A, anticoagulant aptamers have now been generated against most of the coagulation proteases. These aptamers have been shown to be selective, bind tightly to their target proteins, and act as anticoagulants by interfering with the protein-protein interactions that are central to the coagulation cascade. Thus, aptamers may prove to be valuable tools and analytical reagents for identifying and defining functionally important regions of the coagulation factors. In this regard, aptamers have several advantages over antibodies, including being chemically synthesizable with minimal batch-to-batch variability, having an unlimited shelf life and able to withstand temperature insult, utilizing a relatively quick *in vitro* system for their isolation, being able to be manipulated with ease, and exhibiting binding affinities comparable to antibodies. Unfortunately, to date, the only crystal structures reported in the coagulation field are for aptamers bound to thrombin<sup>7, 23, 71</sup> and VWF<sup>8</sup>, and aptamers as a whole have yet to be universally adopted as reagents<sup>72</sup>. To this end, additional structural and biochemical information is greatly needed to further elucidate the mechanisms aptamers employ to bind their target proteins, and this information could yield insight into exosite functions for the entire coagulation system.

As therapeutics, aptamers have received significant attention as rapid onset anticoagulants, with aptamers to thrombin, FIX/FIXa, and VWF evaluated in clinical studies (Table 2)<sup>13, 16, 17, 41, 44</sup>. Overall, coagulation aptamers make up four out of the ten aptamers assessed in clinical trials to date<sup>6</sup>. While the disease states of these other aptamers range from cancer to diabetes, one aptamer, Macugen (pegaptanib), targeting vascular endothelial growth factor, has been approved for use in age related macular degeneration since 2004 and another aptamer, Fovista (E10030), targeting platelet derived growth factor, is rapidly progressing through definitive phase 3 studies for the same indication (NCT01944839). The majority of the other clinically tested aptamers are in phase 2 or awaiting phase 3 clinical trials, highlighting that the field has increasingly excelled at overcoming challenges inherent in defining a new class of drugs. The recent results from the clinical studies of anticoagulant aptamers are encouraging from the perspective of defining a novel class of rapidly reversible anticoagulant agents. While gains have been made, the recent issues with the unexpected effects of the TFPI aptamer, as well as the rare allergic responses to the FIX/FIXa aptamer peginacogin, highlight the need for continued effort so that additional translation of these promising agents can proceed.

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### Significance

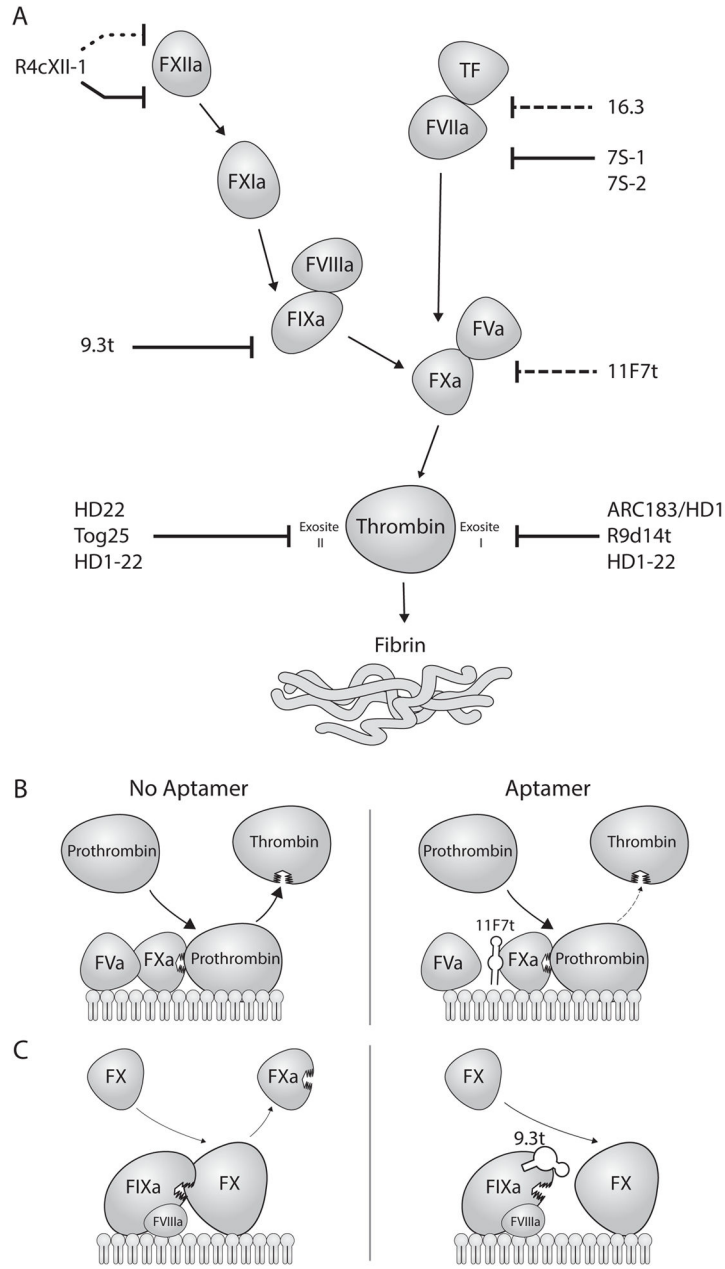
Aptamers are a novel, emerging class of therapeutics that distinguish themselves from other classes of drugs for several reasons: their ability to specifically target a wide range of proteins, their ease of isolation, their chemical flexibility and synthesis, and their inherent ability to have their activities reversed with an antidote. This latter property lends itself well to anticoagulation, as maintaining a balance between bleeding and thrombosis is critical for patient care. Aptamers have been developed that target many proteases of the coagulation cascade, and studies into the mechanisms of aptamer inhibition determine that this class of drugs mainly mediates anticoagulation by binding to exosites and inhibiting protein-protein interactions. While these aptamers have been used to probe protein function, several of these anticoagulant aptamers have also been tested in clinical trials.

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**Figure 1. Mechanisms of aptamer inhibition of the coagulation cascade**

A. Aptamers inhibit the serine proteases of the coagulation cascade by binding to exosites and either disrupting cofactor binding (long dashed line), downstream zymogen binding (solid line), or activation of itself (short dashed line) leading to inhibited or reduced activation of the downstream zymogen, and thus reducing fibrin formation. B. An example of an aptamer (11F7t) disrupting cofactor binding by inhibiting FVa to bind to FXa, leading to a reduction in the amount of thrombin formed. C. An example of an aptamer (9.3t) disrupting zymogen binding by inhibiting FX binding to FIXa, leading to an inhibition of FX activation. FIXa binding to its cofactor FVIIIa is still intact.

**Table 1**

Characteristics of aptamers targeting the coagulation cascade.

Name	Target	Oligonucleotide	Binding Affinity	Mechanism of Action
ARC183/HD1	FIIa	DNA	7.1nM	Inhibits pro/exosite I <sup>31</sup>
HD22	FIIa	DNA	2.4nM	Inhibits exosite II <sup>31</sup>
HD1-22	FIIa	DNA	0.65nM	Inhibits pro/exosite I and exosite II <sup>31</sup>
Tog25	FII	RNA	3nM	Inhibits exosite II <sup>32</sup>
R9d14t	FII/FIIa	RNA	10nM/1nM	Inhibits pro/exosite I <sup>34</sup>
11F7t	FXa	RNA	1.1nM	Blocks FXa/FVa assembly <sup>36</sup>
16.3	FVIIa	RNA	11.3nM	Inhibits TF/FVIIa assembly <sup>37</sup>
7S-1/7S-2	FVII	RNA	65.7nM/59.2nM	Unknown <sup>38</sup>
9.3t	FIXa	RNA	0.58nM	Inhibit FIXa-mediated activation of FX without affecting FIXa/FVIIIa complex assembly <sup>14, 39</sup>
R4cXII-1	FXII/FXIIa	RNA	8.9nM/0.5nM	Inhibits autoactivation of FXIIa and FXIIa-mediated activation of FXI <sup>54</sup>

**Table 2**  
**Clinical trials of aptamers targeting proteins involved in coagulation**

Data from [www.clinicaltrials.gov](http://www.clinicaltrials.gov). TTP = thrombotic thrombocytopenic purpura, VWD = von Willebrand Disease

Aptamer (Sponsor) Target	Condition	Trial Phase	ClinicalTrials.gov Identifier and References
NU172 (ARCA Biopharma, Inc.) Thrombin	Heart Disease	Phase 2 Recruitment Status Unknown (June 2011)	NCT00808964
	Healthy	Phase 1 Completed (Oct 2005)	NCT00113997 <sup>13</sup>
REG1 (Regado Biosciences, Inc.) FIX/FIXa aptamer/antidote system	Coronary Artery Disease	Phase 2 (REVERSAL-PCI) Completed (Oct 2008)	NCT00715455 <sup>17</sup>
	Acute Coronary Syndrome	Phase 2 (RADAR) Completed (Jan 2011)	NCT00932100 <sup>43, 44</sup>
	Coronary Artery Disease	Phase 3 (REGULATE-PCI) Terminated (Oct 2014)	NCT01848106
REG2 (Regado Biosciences, Inc.) FIX/FIXa aptamer/antidote system	Healthy (Subcutaneous Delivery)	Phase 1 Completed (Dec 2009)	NCT01872572 <sup>51</sup>
	Thrombosis	Phase 1 Completed (March 2007)	NCT00432770 <sup>64</sup>
ARC1779 (Archemix Corp.) Von Willebrand Factor	TTP, VWD-Type 2B	Phase 2 Completed (Dec 2008)	NCT00632242 <sup>67, 68</sup>
	Acute Myocardial Infarction	Phase 2 Terminated (Jan 2009)	NCT00507338
	VWD	Phase 2 Withdrawn (Aug 2009)	NCT00694785
	Thrombotic Microangiopathy, TTP	Phase 2 Terminated (Nov 2009)	NCT00726544 <sup>66</sup>
	Intracranial Embolism, Cerebral Thromboembolism, Carotid Stenosis	Phase 2 Terminated (Jan 2010)	NCT00742612 <sup>73</sup>
ARC19499 (Baxalta US Inc.) TFPI	Hemophilia	Phase 1 Terminated (Dec 2011)	NCT01191372 <sup>62</sup>