above) for 30 min at 25 °C. Mixtures were passed over nitrocellulose filters under vacuum. Filters were washed with 3 ml of binding buffer before RNA recovery.

Other methods

We carried out ultraviolet crosslinking, immunoprecipitation, RNase H treatment and U1C-depletion as described[11,15].

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Competing interests statement

The authors declare that they have no competing financial interests.

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RNA aptamers as reversible antagonists of coagulation factor IXa

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Many therapeutic agents are associated with adverse effects in patients. Anticoagulants can engender acute complications such as significant bleeding that increases patient morbidity and mortality[1]. Antidote control provides the safest means to regulate drug action. For this reason, despite its known limitations and toxicities, heparin use remains high because it is the only anticoagulant that can be controlled by an antidote, the polypeptide protamine[2–4]. To date, no generalizable strategy for developing drug–antidote pairs has been described. We investigated whether drug–antidote pairs could be rationally designed by taking advantage of properties inherent to nucleic acids to make antidote-controlled anticoagulant agents. Here we show that protein-binding oligonucleotides (aptamers) against coagulation factor IXa are potent anticoagulants. We also show that oligonucleotides complementary to these aptamers can act as antidotes capable of efficiently reversing the activity of these new anticoagulants in plasma from healthy volunteers and from patients who cannot tolerate heparin[5]. This generalizable strategy for rationally designing a drug–antidote pair thus opens up the way for developing safer regulatable therapeutics.

To determine if properties inherent to nucleic acids can be used to develop an antidote-controlled anticoagulant, we first sought to generate an aptamer with anticoagulant activity. Procoagulant proteins that promote fibrin clot formation have been targeted in the development of many anticoagulant agents[6], and anticoagulant aptamers have been isolated against coagulation factors VIIa[7] and thrombin[8–10]. Here we describe the isolation of aptamers specific for coagulation factor IXa (FIXa).

We employed iterative *in vitro* selection techniques[11,12] to screen a nucleic-acid-based combinatorial library containing about 10^{14} species for those members capable of binding FIXa with high affinity. To ensure that the resultant aptamers would be stable in human plasma, the starting library contained 2′-fluoropyrimidines[13]. *In vitro* selection was performed for eight rounds against FIXa. The RNAs present in the round-eight library were converted to complementary DNAs and sequenced. Sixteen of the RNAs obtained bind FIXa, and all share a conserved primary sequence and secondary structure (Fig. 1a, b). Of these RNAs, aptamer 9.3 bound FIXa with the highest affinity (dissociation constant $K_d = 0.65 \pm 0.2$ nM). Covariation analysis of this sequence family[14] aided in the generation of a truncated version of this aptamer, termed 9.3t ($K_d = 0.58 \pm 0.1$ nM), and an inactive mutant version, 9.3tM (K_d for FIXa $> 10\,\mu$M) (Fig. 1b). Aptamer 9.3t exhibits greater than 5,000-fold specificity for FIXa versus the structurally similar coagulation factors VIIa, Xa, XIa and activated protein C (K_d values $> 5\,\mu$M). Attachment of a polyethlyene glycol of relative molecular mass $M_r = 40{,}000$ to the 5′ end of an aptamer has been shown to enhance the bioavailability of aptamers *in vivo*[15,16], and its attachment to aptamer 9.3t had a nominal impact on the affinity of this aptamer for FIXa, (K_d of Peg-9.3t, 2.83 ± 0.4 nM).

We next determined if aptamer 9.3t inhibits FIXa activity. *In vivo*, FIXa forms a complex with coagulation factor VIIIa on a cell surface

and catalyses the cleavage of FX[17]. Therefore, we pre-assembled the FIXa–FVIIIa enzyme complex on a liposome surface, and measured the ability of aptamer 9.3t to inhibit the activation of FX by this complex (Fig. 2a). Aptamer 9.3t, but not 9.3tM, completely blocked FX cleavage by the FIXa–FVIIIa enzyme complex. Next, we determined if aptamer 9.3t could block the activity of FIXa in human plasma (Fig. 2b). Specific inhibitors of FIXa prolong the clotting time of plasma as measured in activated partial thromboplastin time (APTT) assays, but do not prolong the clotting time of plasma when measured in prothrombin time (PT) assays[17]. Aptamers 9.3t and Peg-9.3t prolonged the APTT clotting times in a dose-dependent manner, but had no effect on clotting times measured in PT assays, demonstrating that the aptamers specifically inhibit the FIXa pathway in plasma. Both aptamers were capable of increasing the APTT clotting time to the level observed in plasma from individuals deficient in FIX. By comparison, control aptamer 9.3tM had no effect in either clotting assay.

FIXa has been described as a 'safe' target for anticoagulation and antithrombotic therapy because anti-FIXa agents have exhibited reduced bleeding risks in animal models when used at their minimal effective doses as compared to heparin[18–20]. However, at marginally higher doses, the same agents have exhibited bleeding profiles no different from that of heparin[18–20]. Such an experience in well-controlled animal studies suggests that in the clinical setting, tight regulation of FIXa inhibitors would enhance their safety and facilitate their medical use. Antidote control is preferable to other methods of drug regulation such as pharmacokinetic strategies, as patient pathophysiology can significantly affect the rate of drug clearance. Therefore, we sought to design an antidote that could tightly regulate the anticoagulant activity of aptamer 9.3t. We hypothesized that by altering the shape of an aptamer from an active to an inactive conformation, we could modulate the target binding and inhibitory activity of the aptamer. Moreover, we speculated that any agent that critically alters the conformation of an aptamer could act as an antidote for that aptamer.

To test this hypothesis, we investigated the use of oligonucleotides complementary to aptamer 9.3t as potential antidotes for this anticoagulant (Fig. 3a). We designed a series of antidote oligonucleotides complementary to different portions of aptamer 9.3t (Fig. 3b). To compare the antidote activity of these oligonucleotides, we evaluated them under clinically relevant conditions. First, we anticoagulated human plasma with aptamer 9.3t. Then we added increasing amounts of antidote oligonucleotides to the anticoagulated plasma and measured the plasma's ability to clot 10 min later (Fig. 3c). We observed a wide range in the ability of the various antidote oligonucleotides to reverse the anticoagulant activity of aptamer 9.3t, with antidote 5-2 being the most effective. The relative

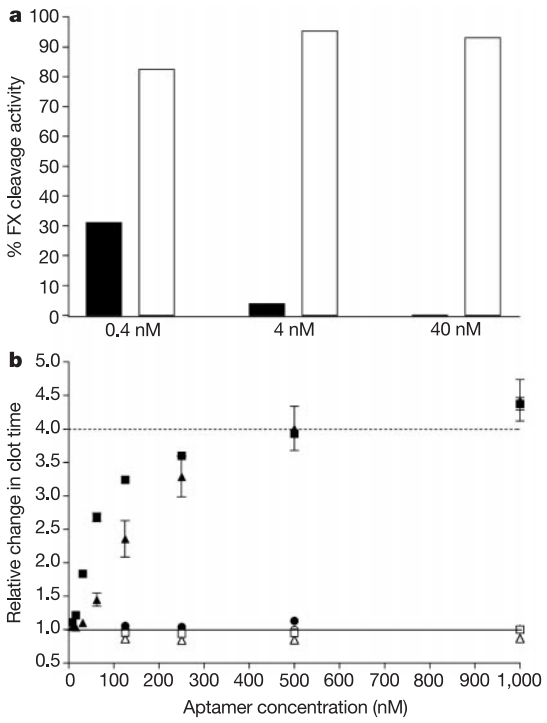

Figure 2 Inhibition of FIXa function by aptamers 9.3t and Peg-9.3t. **a**, The rate of FX activation by the FVIII–FIXa enzyme complex was measured in the presence of varying concentrations of 9.3t (filled bars) or 9.3tM (open bars). **b**, Varying concentrations of aptamers 9.3t, Peg-9.3t or 9.3tM were added to normal human plasma and clotting times were measured in APTT or PT assays: 9.3t APTT (filled squares), PT (open squares); Peg-9.3t APTT (filled triangles), PT (open triangles); 9.3tM APTT (filled circles), PT (open circles). The dashed line indicates the observed increase in the APTT of human plasma deficient in FIX (less than 1% FIX activity) as compared with normal human plasma (solid line). Data shown is the mean ± s.e.m. for three independent measurements.

ability of these antidote oligonucleotides to bind aptamer 9.3t directly correlates with their effectiveness as antidotes in human plasma (Fig. 3d).

An antidote must be able to rapidly reverse an anticoagulant's activity, and dosing of an antidote would be simplified if the antidote's effects were durable. To determine if antidote 5-2 met these criteria, we further characterized its activity against aptamer Peg-9.3t, as the pegylated form of the aptamer is more likely to be used in the clinic. Antidote 5-2 completely reverses the anticoagulant activity of aptamer Peg-9.3t in 10 min and does so at

a

Aptamer		S1	L1	S2	L2	S2	L3	S1	
9-3	5'	GGGAUGGGGA	CU**AUA**CC	GCG	UAAUGC	UGC	C	UCCCCAUUCC	GGAACGCU 3'
9-20	5'	GGGGA	CU**AUA**CCG	GCA	AUCG	UGC	A	UCCCC	UGGACCUAACAAUA 3'
9-19	5'	GGaUGGGA	CC**AUA**	ACGA	CUAC	UCGU	GAA	UCCCACC	AUCAGCGCACAA 3'
9-4	5'	GGGaUGGGC	ACU**AUA**C	GCA	UCU	UGC	U	GCCUGCCC	GCGAGUCAAUUG 3'
9-12	5'	GGGaUGGG	CG**AUA**	UAC	ACAUUG	GUG	AU	CCCACCC	ACAUGAAACCACAG 3'
9-17	5'	GAGGqauGGGA	CC**AUA**C	GCA	CAU	UGC	UGAA	UCCCCCUC	AAUAGCACCUAC 3'
9-25	5'	GGGAUGGGGA	CC**AUA**	ACUC	UAAC	GGGU	GAA	UCCCgCAUCUC	GACAAUA 3'
9-26	5'	GGGAuGGG	UG**AUA**	ACCA	CUC	UGGU	GAA	CCCcUCCC	GACUUGCUCGCA 3'
9-11	5'	GGGaUGGGA	CU**AUA**	UUCGG	AAU	CUGGA	C	UCCCACCU	GCCUGCCCCAGA 3'
9-2	5'	GGGAUGGG	CU**AU**AUA	CAC	GCUG	GUG	AU	CCCAUCUC	AAUUGAAACAACA 3'
9-7	5'	GGAUGGG	CG**AUA**	ACCA	ACA	UGGU	GAU	CCCAUUC	AUCAUACCCUACAA 3'
9-28	5'	GGGAuGGGCG	CC**AUA**C	GCA	CAU	UGC	UGCAU	CGCCUUCCC	GUAAGAAC 3'
9-16	5'	GAGGGaUGGG	CC**AUA**C	GUGG	ACGA	CUGC	A	CCCGaCCCUU	CAGCCCAGGUC 3'
9-18	5'	GGGaUGGG	CC**AUA**	ACCA	CUU	UGGU	GAA	CCCACCC	AGCUCCUGUGAUUG 3'
9-14	5'	GGGAuGGGGA	CU**AUA**	CGU	GAACG	ACU	GCA	UCCaCUUCCC	CGCCAUGG 3'
9-27	5'	GGGaUGGG	UA**AUA**	ACU	GUA	UGG	UGAA	CCCACCC	AAACUCCCAUGGCUA 3'

b

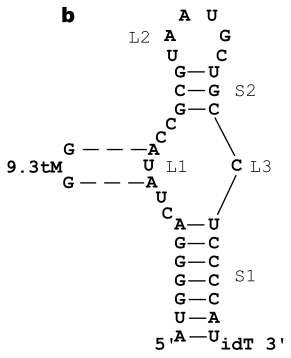

Figure 1 Sequences and secondary structure of aptamers isolated against FIXa. **a**, Sequences are aligned on the basis of their predicted secondary structure and listed in order of decreasing affinity for FIXa. Shown are sequences that originated from the randomized region of the library used in the SELEX process and portions of the 5′ fixed region sequence proposed to participate in the conserved secondary structure (fixed-region sequences are underlined, conserved loop sequence is in bold) stem (S), loop (L). For some of the sequences, mis-pairings are present in stem 1 (S1), and are denoted by lower-case letters. **b**, Predicted secondary structure of aptamer 9.3t, a 35-nucleotide truncate of aptamer 9.3 (idT, inverted deoxythymidine). A double-point mutation that yields an inactive aptamer, 9.3tM, is also shown.

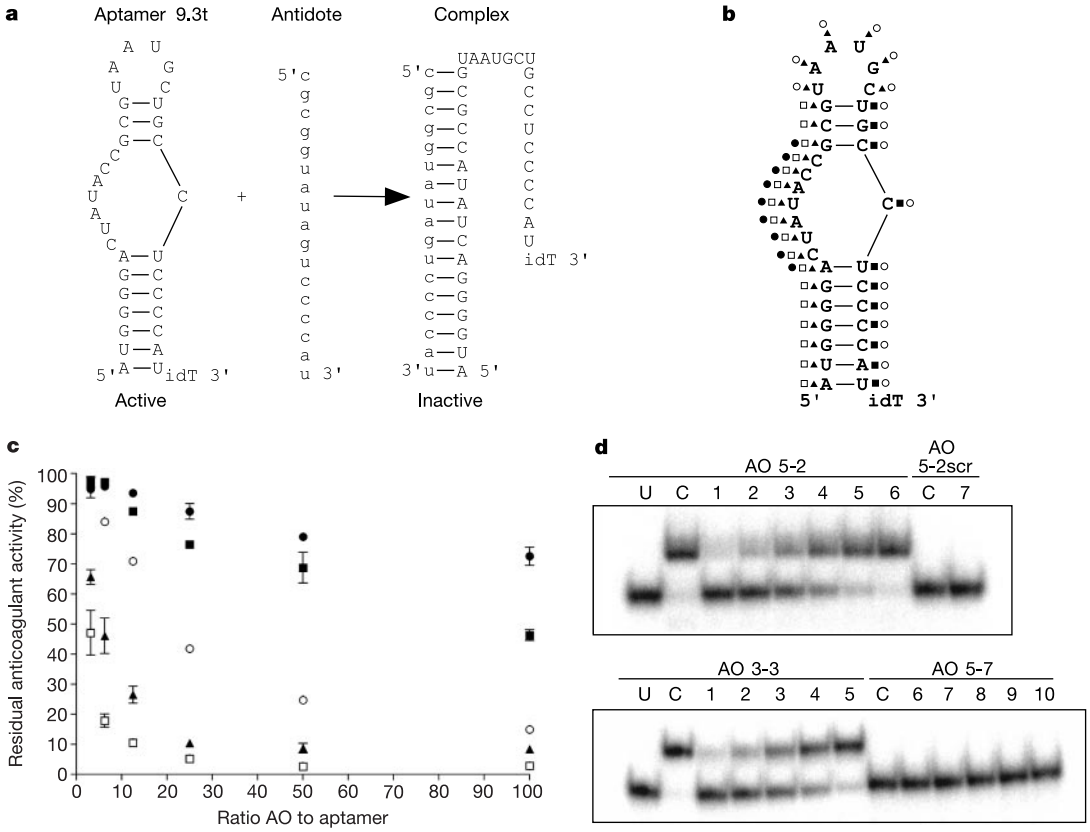

Figure 3 Antidote oligonucleotides to aptamer 9.3t. **a**, Cartoon depicting an antidote oligonucleotide binding to aptamer 9.3t to form an inactive aptamer–antidote complex. **b**, Antidote oligonucleotides used in this study: 5-1 (filled triangles), 5-2 (open squares), 5-7 (filled circles), 3-1 (open circles), 3-3 (filled squares). Placement of the symbols depicts the positions of complementarity between the antidote and the aptamer. All antidote oligonucleotides are 2′-Omethyl modified. **c**, Reversal of anticoagulant activity of aptamer 9.3t (50 nM) by antidote oligonucleotides (AO) in human plasma. Data are the average ± range for duplicate measurements. **d**, Native gel analysis of antidote oligonucleotide binding to aptamer 9.3t. Untreated aptamer (U), aptamer denatured and complexed with antidote before gel loading (C). The upper panel shows the antidote molar ratio to aptamer increasing from 0.5:1 to 16:1 in twofold increments (lanes 1–6) and the 16:1 ratio of scrambled control antidote to aptamer (lane 7). The lower panel shows the antidotes molar ratio to aptamer increasing from 1:1 to 16:1 in twofold increments (lanes 1–5 and 6–10).

slightly lower concentrations than required for aptamer 9.3t (Fig. 4a). By comparison, a control 'scrambled' version of antidote 5-2 (5-2scr) did not affect the anticoagulation activity of the aptamers or the clotting activity of the plasma. To evaluate the kinetics of onset and persistence of antidote action, we measured the time required for antidote 5-2 to reverse the anticoagulant activity of aptamer Peg-9.3t and the durability of this reversal once established. The antidote activity of oligonucleotide 5-2 towards aptamer Peg-9.3t is rapid, and concentration dependent in human plasma (Fig. 4b). Finally, after its addition, antidote 5-2 reversed the aptamer's activity for over 5 h, indicating that the activity of this antidote is effectively irreversible in human plasma (Fig. 4c). In addition, plasma treated with aptamer Peg-9.3t but given no antidote remained anticoagulated over the time course of this experiment, demonstrating that aptamer Peg-9.3t is quite stable in human plasma, as expected for an RNA containing 2′-fluoropyrimidine modifications and an inverted deoxythymidine on its 3′ end[21].

The ability to control the anticoagulant activity of heparin with protamine enables safer treatment of patients undergoing procedures requiring a high level of anticoagulation, in whom the post-procedural risk of haemorrhage is high. However, approximately 3–5% of the estimated 12 million patients who receive heparin each year develop a drug-induced immunologic response, termed heparin-induced thrombocytopenia (HIT)[22], that contraindicates further treatment of these patients with heparin[5]. Alternative anticoagulants are available for treatment of these patients, but haemorrhagic complications and recurrent life- and limb-threatening thromboembolism are common during treatment[23,24]. Moreover, none of these anticoagulants can be neutralized by a reversing agent, which limits physician control during treatment of these patients. Therefore, we investigated the ability of aptamer Peg-9.3t and antidote 5-2 to serve as an anticoagulant–antidote pair in plasma samples from six patients with HIT, three with end-stage renal disease requiring haemodialysis and thus repeated anticoagulation, and three with thromboembolic complications requiring anticoagulant therapy. Aptamer Peg-9.3t prolonged the APTT clotting times of plasma from all six patients, and antidote 5-2 was able effectively to reverse this anticoagulant activity to the pretreatment baseline of each patient (Fig. 5). Importantly, two patients were receiving anticoagulant therapy at the time samples were taken (patient 3 on danaparoid sodium and patient 6 on warfarin), demonstrating that in patient plasma, the aptamer–antidote pair can function independently of an 'on board' anticoagulant.

We have demonstrated that drug–antidote pairs can be rationally designed by taking advantage of properties inherent to nucleic acids to make an antidote-controlled anticoagulant aptamer. To achieve this end, we adopted an approach employing complementary antidote oligonucleotides, as this approach is straightforward and generalizable to any aptamer. We have generated an antidote oligonucleotide that effectively, rapidly and durably inactivates a new anticoagulant aptamer in human plasma under parameters set as the clinical standard by heparin and protamine[3]. Moreover, the demonstration that aptamer Peg-9.3t and its antidote function in

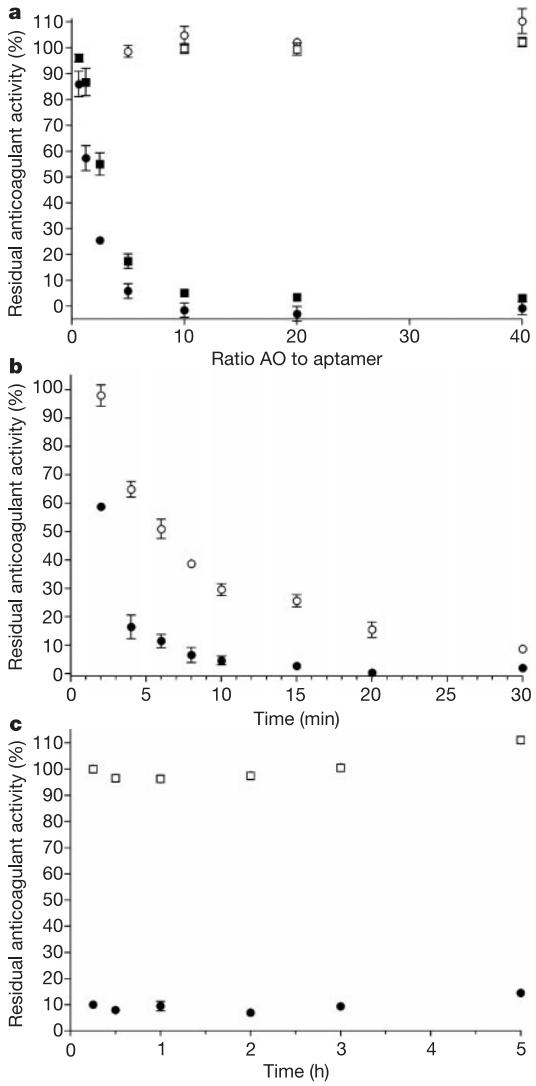

Figure 4 Properties of antidote oligonucleotide 5-2. **a**, The ability of antidote oligonucleotides 5-2 or 5-2scr to reverse the anticoagulant activity of aptamers 9.3t and Peg-9.3t (aptamers at 125 nM) was measured in pooled human plasma: 9.3t + 5-2 (filled squares); 9.3t + 5-2scr (open squares); Peg-9.3t + 5-2 (filled circles); and Peg-9.3t + 5-2scr (open circles). Data is mean ± s.e.m. for three independent measurements. **b**, Kinetics of the reversal of the anticoagulant activity of aptamer Peg-9.3t by antidote oligonucleotide 5-2 in human plasma, Peg-9.3t + 2 × 5-2 (open circles), +10 × 5-2 (filled circles). Data is average ± range for two independent measurements. **c**, The duration of the reversal of the anticoagulant activity of aptamer Peg-9.3t by antidote oligonucleotide 5-2 was measured in human plasma (filled circles). The persistence of the anticoagulant activity of Peg-9.3t without the antidote in human plasma is shown for comparison (open squares). Data is the average ± the range for two independent measurements.

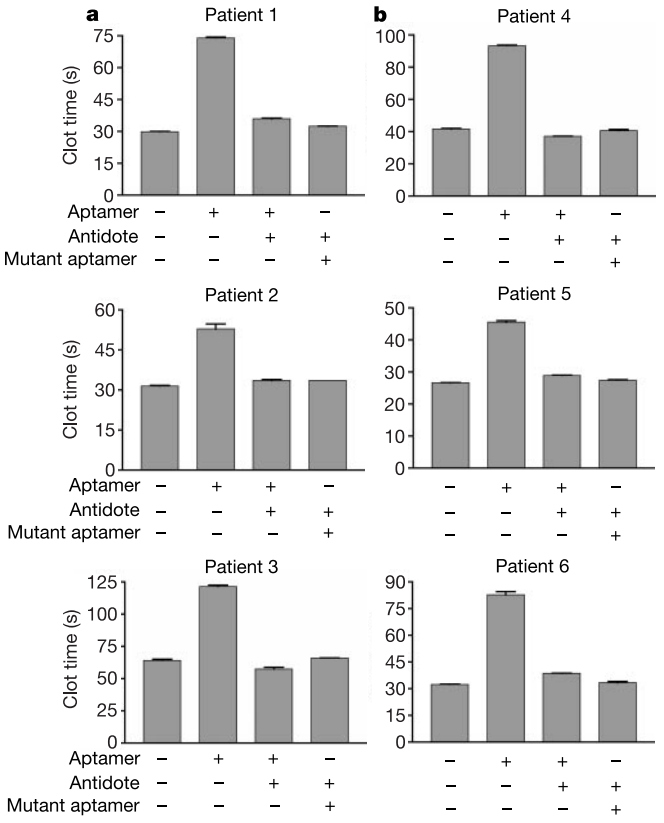

Figure 5 Antidote-controlled anticoagulation of plasma from patients with HIT. **a**, **b**, The activity of aptamer Peg-9.3t and antidote 5-2 were tested in plasma from haemodialysis-dependent patients diagnosed with HIT (**a**) and in plasma from patients suffering from thromboembolic complications of HIT (**b**). Plasma samples were treated as indicated: aptamer, 125 nM Peg-9.3t; antidote, 1.25 μM AO 5-2; and mutant aptamer, 125 nM 9.3tM. Data is reported in seconds (s) and is the average ± range of duplicate measurements.

plasma from patients suffering from HIT is a first step towards providing such an anticoagulant–antidote pair for this patient population. The relative excess of antidote over aptamer necessary to achieve these results is compatible with the use of this approach *in vivo*, and current efforts are aimed at translating these results obtained with patient samples into animal models. Over the past decade much progress has been made in translating aptamers into therapeutics, including the demonstration of efficacy of several aptamers in animal models, and phase III testing of an aptamer against vascular endothelial growth factor in clinical trials[21,25]. However, the rationale for therapeutic aptamer development has been questioned because other classes of antagonists, such as humanized monoclonal antibodies, have already proved useful as therapeutic agents[26]. The ability to rationally design antidotes to alter the shape and activity of aptamers is unique to aptamers. We believe this property distinguishes aptamers from other classes of antagonists, such as antibodies, peptides or small molecules. We recommend the clinical development of safer regulatable therapeutics based upon aptamer–antidote pairs for indications in which the acute side effects of treatment may lead to increased morbidity and mortality. □

Methods

Generation of aptamers

In vitro selection procedures were carried out as previously described[7,10,27]. The sequence of the starting RNA combinatorial library was 5′-GGGAGAGAGGAAGAGGGAUGGG-N_{40}-CAUAACCCAGAGGUCGAU-3′, where N_{40} represents 40 contiguous nucleotides with equimolar A, G, C and U at each position. 2′F cytidine triphosphate and uridine triphosphate were purchased from TriLink Biotechnologies and incorporated into RNA libraries by *in vitro* transcription as described[10]. The selection was carried out in selection buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$, and 0.01% bovine serum albumin (BSA) at 37 °C, using filtration through nitrocellulose membranes to separate FIXa-RNA complexes from unbound RNA.

Binding studies

K_d values were determined using double-filter nitrocellulose filter binding assays[7]. All binding studies were performed in physiologic binding buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, and 0.01% BSA) at 37 °C, and all coagulation factors were purchased from Haematologic Technologies Inc. Aptamer 9.3t, 9.3tM and Peg-9.3t were synthesized and purified by Dharmacon Research Inc. K_d values of aptamers 9.3 and 9.3t binding to FIXa and other coagulation factors were determined in direct binding assays, and K_d values for aptamers Peg-9.3t and 9.3tM binding to FIXa were determined in competition binding assays. Competition binding studies were performed and results

analysed as described[10], with a final FIXa concentration of 1 nM. All data presented is the mean ± s.e.m. for at least three independent experiments.

Enzyme assays

Factor VIII (Hemophil M, Baxter Healthcare) was activated with thrombin, then the FVIIIa solution was added to factor IXa and phospholipid vesicles (lipids purchased from Avanti Polar Lipids and vesicles prepared as in ref. 28) and allowed to form the FIXa–FVIIIa enzyme complex. Factor X and aptamer were then added, and factor X activation measured with a chromogenic substrate (Pefachrome fXa, Centerchem Inc.). Final concentrations were: factor IXa, 0.2 nM; factor VIIIa, 0.7 nM; lipid, 40 μM; factor X, 135 nM; and factor Xa substrate, 1 mM. The rate of factor Xa generation was determined by a second-order fit to the data. The %FX cleavage activity is 100 times the rate of FXa generation in the presence of aptamer divided by rate in the absence of aptamer.

Clotting assays

Activated partial thromboplastin time (APTT) assays were performed using a model ST4 mechanical coagulometer (Diagnostica Stago Inc.). Aptamer in binding buffer (5 μl) without BSA or binding buffer without BSA alone was added to pooled normal human plasma (50 μl) (George King Biomedical), and incubated for 5 min at 37 °C. MDA platelin (50 μl) (bioMerieux) was then added and allowed to activate the plasma for 5 min, followed by the addition of 25 mM $CaCl_2$ (50 μl) to initiate the clotting reaction. Data is expressed as the relative change in clot time; the clot time in the presence of aptamer divided by the clot time in the presence of buffer alone. All reactions were performed in duplicate, and only duplicates differing by <10% were used in analysis.

Prothrombin time (PT) clotting assays were performed as previously described[7] except that 5 μl of aptamer was added to 50 μl of normal pooled human plasma, and all reactions were carried out at 37 °C.

Antidote assays

Antidote oligonucleotides were synthesized and purified by Dharmacon Research, Inc. Antidote activity was measured 10 min after antidote addition to plasma containing aptamer in APTT clotting assays. Briefly, human plasma was anticoagulated with aptamer, antidote oligonucleotide (5 μl) was added and the incubation continued for 5 min before the addition of MDA platelin. Antidote activity is expressed as the per cent residual anticoagulant activity T of the aptamer, which is:

$$[1 - (T_{\text{aptamer alone}} - T_{\text{aptamer+antidote}})/(T_{\text{aptamer alone}} - T_{\text{baseline}})] \times 100.$$

For measuring the kinetics of the onset of antidote activity, the incubation time of the plasma following MDA platelin addition was reduced to 1 min to allow for shorter timepoints to be measured. This increased the baseline APTT from 30.2–32.5 s to 34.2–36.8 s.

Gel shift assays were performed essentially as described[29]. Aptamer 9.3t (50 nM with trace ^{32}P-labelled) was incubated for 10 min with varying concentrations of antidote oligonucleotide in binding buffer without BSA before loading on a 12% polyacrylamide gel containing 2 mM $CaCl_2$. Gels were run for 3 h at 300 V and visualized using a Storm 840 Phosphorimager (Molecular Dynamics).

Patient samples

Plasma samples from six patients with HIT were studied. Clinical criteria for the diagnosis of HIT included thrombocytopenia and/or new or recurrent thrombosis after five or more days of heparin therapy[5]. Serologic criteria included a positive heparin-induced platelet aggregation assay and/or elevated heparin/platelet factor 4 antibody levels detected by enzyme-linked immunosorbent assay (ELISA) (GTI Inc.). Five patients met both clinical and serologic criteria; one patient fulfilled clinical criteria but had negative serologic studies. The Institutional Review Board at Duke University Medical Center approved these studies, and informed consent was obtained from all patients.

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Competing interests statement

The authors declare competing financial interests: details accompany the paper on *Nature*'s website (http://www.nature.com/nature).

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corrigendum

Sub-ångstrom resolution using aberration corrected electron optics

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For this Letter the disclosure form for the declaration of competing financial interests was incorrectly filled out because of a misunderstanding. The statement should have read: 'The authors declare competing financial interests: details accompany the paper on *Nature*'s website (http://www.nature.com/nature).' The details on the website should have read: 'O.L.K. and N.D. have a personal financial interest in Nion, R&D.'. We (the authors) had no intention of misrepresenting the origin of the work. □