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Translating Nucleic Acid Aptamers to Antithrombotic Drugs in Cardiovascular Medicine

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Abstract Nucleic acid aptamers offer several distinct advantages for the selective inhibition of protein targets within the coagulation cascade. A highly attractive feature of aptamers as antithrombotics is their ability to encode for complementary "controlling agents" which selectively bind to and neutralize their active counterparts via Watson–Crick base pairing or, in a less selective and clinically characterized manner, cationic polymers that can counteract the activity of an aptamer or free/protein-complexed nucleic acid. The former property allows aptamer-based antithrombotic therapies to be administered with a goal of selective, high intensity target inhibition, knowing that rapid drug reversal is readily available. In addition, by purposefully varying the ratio of active agent to a specific controlling agent administered, the intensity of antithrombotic therapy can be regulated with precision according to patient needs and the accompanying clinical conditions. REG1, currently undergoing phase 2B clinical investigation, consists of an RNA aptamer (RB006; pegnivacogin) which targets factor IXa and its complementary controlling agent (RB007; anivamersen). Aptamers directed against other serine coagulation proteases, some with and some without parallel controlling agents, have been designed. Aptamers directed against platelet surface membrane receptor targets are in preclinical development. The following review offers a contemporary summary of nucleic acid aptamers as a translatable platform for regulatable antithrombotic drugs expanding the paradigm of patient- and disease-specific treatment in clinical practice.

Keywords Nucleic acids · Aptamers · Antithrombotic agents

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Introduction

The fields of cardiovascular medicine and hematology have many things in common, including the frequent management of patients with thrombotic disorders. Accordingly, considerable time, thought, and effort have been dedicated by clinicians and scientists alike to define the ideal antithrombotic strategy. To date, however, the effectiveness of antithrombotic therapy on clinical outcomes is limited in large part by the attendant bleeding which accompanies these therapies, with bleeding risk closely coupled to effectiveness in preventing ischemic events [1–3].

Two strategies have emerged as possibly altering the safety profile of an antithrombotic therapy for any given level of potency. One is to target novel points of enzymatic interfaces in the coagulation process, thereby potentially altering bleeding risk in a favorable direction [4]; however, selection of a target must be based soundly on a firm understanding of biology and biochemistry as they apply to thrombosis and hemostasis.

A second more broadly applicable strategy is the development of potent antithrombotics with either a very short half-life or preferentially which could be actively controlled, allowing high levels of anticoagulation when needed (e.g., in patients with an acute thrombotic events, or during cardiopulmonary bypass), followed by rapid and graded titration of effect once the acute need has been satisfactorily addressed or there is a clinical need for rapid reversal. This "safety net" offered by predictable and reliably drug reversal allows for the administration of doses of the active drug which may consistently inhibit the target to a high degree, minimizing intrapatient response variability.

Aptamers: Defining Features

Protein-binding oligonucleotides or aptamers are single-stranded nucleic acids that inhibit a selected target protein by assuming a highly specific three-dimensional structure that confers high binding specificity and affinity. Like peptides, oligonucleotide libraries can be synthesized based on random selection of their individual building blocks. Individual constituents can be selected based on binding to a target using an iterative purification and enrichment process discovered by Tuerk and Gold and termed systemic evolution of ligands by exponential enrichment (SELEX) [5].

The term aptamer, meaning shape, was chosen by Ellington and Szostak [6] to describe the nucleic acid ligands isolated using this iterative purification process based on their conformational specificity. In contrast to peptides, aptamers have unique advantages including minimal toxicities, low immunogenicity, and easy formulation as either intravenous (IV) or subcutaneous (SC) injectables. Aptamers can also be cost-effectively manufactured using solid-phase chemical process, offering tight quality control of the finished product. Via modification of either the sugar or phosphate components of the phosphodiester backbone, aptamer half-life and serum stability can be finely tuned.

One defining feature of aptamers is that their inherent oligonucleotide sequence encodes for their own controlling agents. Thus, it is relatively straightforward, once an aptamer with appropriate binding specificity has been identified, to obtain the primary sequence and construct a complementary controlling agent designed to form Watson–Crick base pairs with the active agent. This "controlling agent", by irreversibly binding to and altering the conformation of the active aptamer, can specifically neutralize an individual aptamer activity. A potentially novel field of study that requires further investigation is the employment of polymers to neutralize free or protein-complexed nucleic acids in plasma.

Not surprisingly, aptamers have been applied to the clinical investigation of regulatable antithrombotic drugs, where their reversible capabilities might prove especially attractive. Herein, we provide a comprehensive review of oligonucleotide aptamers as a platform for discovery and management approaches to address the needs of patients with thrombotic disorders.

Discovery and Technology Platforms

The initiating substrate for aptamer generation is a combinatorial or shape library composed of single-stranded nucleic acids (RNA, DNA, or modified RNA) composed of random sequences of oligonucleotides. Using a library containing a 40-nucleotide randomized region, for instance, it is possible to generate a library of 1×10^{24} sequences, of which 1×10^{15} are typically screened in practice. Such libraries provide a vast array of shapes, with a complexity $\sim1\times10^{6}$-fold greater than a typical antibody or phage display library. In order to isolate high-affinity nucleic acid ligands from such libraries, a purification and enrichment process known as SELEX (Fig. 1) [5] is employed to enable the identification of oligonucleotides with properties of particular interest [7]. Furthermore, the specificity of the selected molecule permits normal functionality of non-targeted proteins [8].

Aptamers isolated from initial screens can be applied to target structure–function studies and in vitro target validation studies to verify specificity, target inhibition, kinetics of binding, and other biophysical properties.

For therapeutic development of such aptamers, post-selection optimization steps are typically employed to increase the ease of chemical synthesis and facilitate in vivo experiments by improving the molecule's pharmacokinetic properties. These modifications include reducing aptamer length to 40 nucleotides or less, enhancing stability

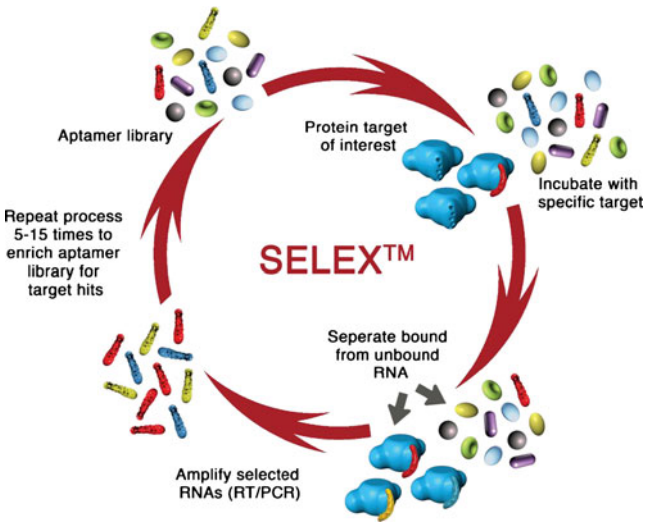

Fig. 1 SELEX technology

via ribonucleotide substitution (2′ modified RNA aptamers are stable in plasma, serum, and other biological fluids), protecting from exonuclease digestion through 3′ end capping and backbone modification, and attenuating renal clearance by adding a polyethylene glycol carrier [9, 10]. Depending on the selected clinical indication, an aptamer can be readily designed to have half-life of minutes to days.

Over the past two decades, the SELEX process has proven to be a robust methodology for the discovery of inhibitors to a wide variety of proteins [5, 6, 11]. However, there are several limitations inherent to the aptamer discovery and development process. First, while SELEX has been successfully carried out utilizing complex proteins mixtures, including membrane-bound targets, the likelihood of success is greatly increased by the availability of high purity, soluble protein as the target for library enrichment. This generally limits the scope of aptamer targets to soluble proteins, receptors that can be prepared as functional extracellular domains, or to peptide fragments of receptors if they can recapitulate the active protein fold. Secondly, as is often the case with monoclonal antibodies (mAbs), the high specificity of the aptamer to the target protein used in SELEX can yield aptamers with limited cross-reactivity to the homologous target from other species. This necessitates the conduct of a thorough cross-species reactivity screen to ensure the candidate aptamer has sufficient pharmacologic activity in at least one non-human species to allow for the conduct of Investigational New Drug-enabling preclinical safety assessment [12]. Finally, as with other classes of oligonucleotide therapeutics, delivery of aptamers across cellular membranes is challenging, leaving plasma and extracellular proteins as the primary targets to be approached with aptamer technology.

Unique Properties

The resulting aptamers typically demonstrate high target protein affinity (low picomolar (1×10^{-12} M) to low nanomolar (1×10^{-9} M)), while being readily synthesized in high yields due to the high coupling efficiency (>98–99%) of oligonucleotide chemical synthesis. The high-affinity properties are the end result of complementary contacts between functional groups on both the oligonucleotide and the target protein. The three-dimensional arrangement of contact also offers high specificity and discrimination of the target from structurally related proteins [13–15].

Control Agent Development

The intellectual advance that oligonucleotide aptamer-based protein inhibitors encode for their own controlling agents opened the door to the development of highly regulatable and reversible agents [11, 16–18]. Given the relationship of anti-ischemic effectiveness and bleeding, the application of this technology to the development of antithrombotic therapy was identified as an immediate area of need.

The initial design of a controlling agent stems directly from the oligonucleotide sequence of the active component. Several complementary oligonucleotides to portions of the active component are synthesized and tested for both binding to the active component as well as biological activity determined by the ability of a controlling agent to reverse the binding (and antithrombotic effect) of the active component to its target protein. Oligonucleotide control agents are highly specific (there are over one billion possible 15-nucleotide length sequences), and the binding energetics of Watson–Crick base pairing and double helical DNA formation dictate rapid and nearly irreversible binding, resulting in virtual immediate and complete reversal of effect [16, 17, 19]. In addition, given that this is a stochastic event, aptamer reversal is titratable: If a large excess of controlling agent is given, all active aptamer is bound and its activity inactivated, while administration of lower doses of controlling agent results in partial reversal of aptamer activity.

More recently, an investigational strategy of non-specific nucleic acid neutralization has been reported. Oney and colleagues developed a series of polymers that could rapidly neutralize nucleic acids and aptamers in combination [20]. These investigators demonstrated that polycationic polymers could rapidly reverse the activity of eight different aptamers, even though the aptamers had different sequences and targeted different proteins, including four which targeted different coagulation proteases: factors IXa, Xa, VIIa, and thrombin. Moreover, they subsequently demonstrated that the polymer, β-cyclodextrin-containing polycation, was able to fully reverse the activity of an anticoagulant aptamer in swine [20]. Clinical investigation has not yet been undertaken.

Aptamers Targeting Factor IXa

Coagulation factor IXa (FIXa) is a vitamin K-dependent protease that plays a pivotal role in both the initiation and the propagation phases of cell-based coagulation [21, 22]. In this functional model, the level of FIXa activity is a more important determinant of thrombin generation than either FXa or thrombin itself and is the primary facilitator of clot propagation on the surface of activated platelets. Factor IX consists of an amino-terminal Gla domain, a hydrophobic domain, two epidermal growth factor-like domains, an activation peptide domain, and a terminal serine protease domain [23]. The lower levels of plasma FIX compared with more downstream targets in the coagulation cascade

may facilitate the achievement of reliable complete levels of target inhibition [23].

Rationale for the inhibition of FIX stems from a variety of clinical observations, including the association of FIX levels with acute coronary syndromes (ACS) and venous thromboembolism and the reduced cardiovascular mortality observed in hemophilia carriers [24, 25]. Hemophilia B also serves as the clinical corollary to FIX inhibition. These patients have abnormally elevated activated partial thromboplastin times (aPTTs) that correlate with bleeding risk and a degree of FIX loss that is associated with bleeding propensity [26]. While hemophilia B carriers (with ~50% decrease in FIX levels) exhibit normal coagulation parameters and no clinically evident bleeding propensity, patients with higher levels (>90%) of FIX loss are characterized by mild (>1.5× baseline) prolongation of the aPTT. These patients also offer insight into the clinical consequences that might be expected to accompany variable degrees of FIX inhibition (Table 1).

Monitoring FIX Inhibition

FIX deficiency is associated with normal PT but prolonged aPTT coagulation measures. Mixing of FIX depleted with FIX replete plasma allows one to derive a relationship between the level of FIX (as a percentage of normal levels) and the resulting effect on aPTT. Such a curve reveals minimal aPTT prolongation (<1.5× prolongation) until >90% of FIX is depleted or inhibited. An aPTT >2× normal represents >99.8% FIX inhibition, and as near complete (>99.9%) inhibition is achieved, a maximal aPTT prolongation of 2.5× to 3.5× is observed. These results set the stage for the clinical monitoring of efficacy of RB006-mediated FIX inhibition in human subjects via the aPTT.

REG1: an Aptamer Lead Clinical Candidate

The REG1 Anticoagulation System (Regado Biosciences™; Durham, NC, USA) consists of RB006 (pegnivacogin), the active aptamer drug component that inhibits FIXa with high affinity and specificity, and RB007 (anivamersen), an oligonucleotide controlling agent complementary to a portion of RB006 that can effectively bind RB006, neutralizing its anti-FIXa activity (Fig. 2) [27].

A comprehensive preclinical program was used to demonstrate the clinical potential of RB006 and related precursor compounds that led to the development of the current generation molecule. These investigations demonstrated in stepwise fashion that RB006 can (1) effectively inhibit FIX activation in vitro, (2) prolong plasma clotting times in vitro using plasma from humans and other species (an important property for preclinical investigation and drug development), (3) systemically anticoagulate animals following an intravenous bolus injection, (4) reduce bleeding in an animal model after control agent-mediated reversal of RB006 activity, (5) prevent arterial thrombosis in several animal models of vascular injury, (6) replace unfractionated heparin (UFH) in an animal model of cardiopulmonary bypass, and (7) modulate the systemic inflammatory response provoked by extracorporeal circulation more effectively than UFH [16, 17, 28].

Clinical Development Platforms

RB006, the anti-FIX aptamer optimized for clinical use, was developed in stepwise fashion in a series of clinical studies establishing relationships between dosing of both RB006 and RB007 with pharmacokinetic and pharmacodynamic effects. Phase IA was a subject-blinded dose escalation, placebo-controlled study (n=85) using fixed doses of RB006 or placebo followed 3 h later by a bolus of RB007 or placebo [27]. The doses of RB006—15, 30, 60, and 90 mg—were chosen to provide a large margin of safety to the study subjects and based on pharmacodynamic effects observed in vitro, to yield minimal effect (15-mg dose) to near complete FIX inhibition (90 mg dose). At each RB006 dosing level, RB007 was administered at a fixed 2:1 weight-based ratio, representing an approximate 20-fold molar excess and 4-fold more than the minimal amount required in vitro to fully neutralize RB006 and return FIXa activity to baseline.

RB006 produced a reliable and rapid (<5 min) stepwise increase in the aPTT in a dose-dependent manner. The duration of effect was dose dependent, with a return to baseline aPTT at 3, 20–24, and 30 h, respectively, for the 15-, 30-, and 60-mg doses of RB006 alone. By extrapolat-

Table 1 Clinical consequences of factor IX deficiency (or inhibition)

Severity of FIX deficiency	Degree of FIX inhibition (%)	aPTT	Bleeding phenotype
Minimal	<70	Normal	None
Mild	70–95	<1.5× nl	No spontaneous bleeding, minor bleeding after surgery
Moderate	95–98	1.5–2.0× nl	Bleeding after minor injury, significant bleeding post-surgery
Severe	>98	>2× nl	Spontaneous bleeding, bleeding post-surgery, injury, accidents

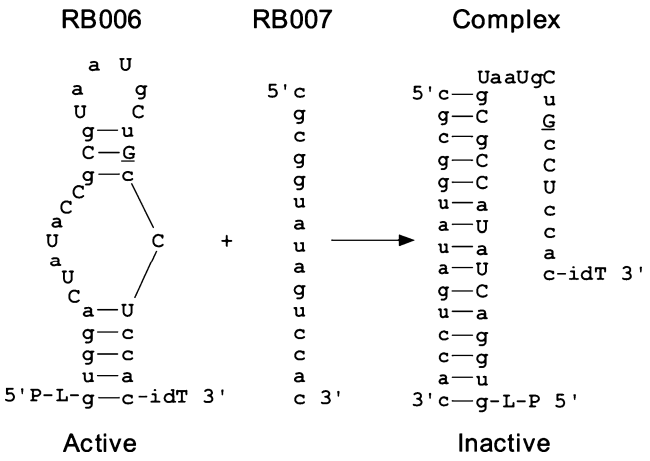

Fig. 2 Structure of RB006/RB007 complex

ing from a FIX activity assay calibration curve, relating FIX levels with aPTT prolongation, the doses of RB006 employed resulted in 35–40%, 80%, 98%, and >99% inhibition of FIXa activity [27].

RB007 administered at a 2:1 weight-based dose resulted in rapid (<1–5 min), complete, and durable (>168 h) return of the aPTT to baseline values [27]. These findings attest to the rapid binding kinetics between RB006 and RB007 and the essentially irreversible binding of the RB006/RB007 complex, resulting in permanent reversal of RB006 effect.

Phase 1B study [29] advanced these findings to patients with stable coronary artery disease (CAD) on aspirin and/or clopidogrel, demonstrating similar pharmacodynamics in this more elderly (ages 50 to 75 years) population taking antiplatelet therapy. RB006 and RB007 were well tolerated, with no major bleeding, serological evidence of complement activation, or other serious adverse events during the 7-day follow-up period. There were five subjects with minor bleeding at peripheral intravenous line sites.

Advanced PK/PD Modeling from Phase I

Because the phase IA and IB studies were performed using fixed doses of RB006, it was possible to derive a full dose–response curve between weight-based RB006 dose and pharmacokinetic effect. The results of the phase 1A and 1B studies were combined, given their similar design, to derive a more complete dose–response curve [29], using a 15-min time point common to both studies.

The relationship between total RB006 dose (Fig. 3a) and weight-based RB006 dose versus relative aPTT prolongation was used to derive a sigmoidal Emax model with the baseline fixed as 1 (i.e., no relative change of aPTT at baseline). These analyses revealed an EC_{50} of 51.7 mg (actual dose) and 0.73 mg/kg (weight-based dose), with an

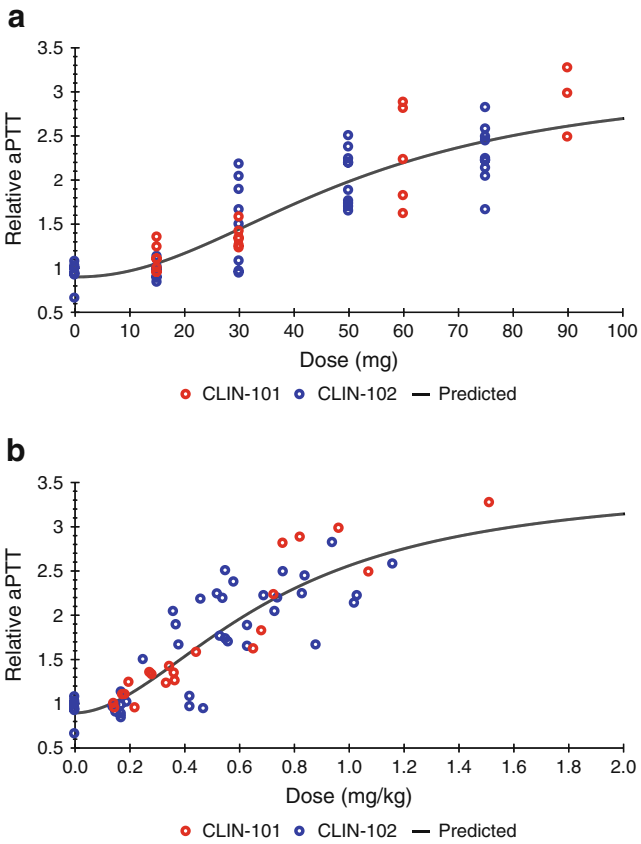

Fig. 3 Relationship between plasma aPTT and RB006 dose in milligrams (**a**) or milligrams per kilogram (**b**)

Emax (representing maximal aPTT prolongation predicted) of 3.15- and 3.5-fold, respectively. Additional PK/PD modeling of achieved RB006 concentrations and simultaneous derived aPTT values demonstrated a calculated EC_{50} of RB006 of 14 μg/ml with a maximal predicted increase in the aPTT to 2.42-fold above baseline.

To gain a better understanding of the relationship between dose and degree of FIX inhibition, FIX inhibition was imputed based on fold increase in aPTT observed in the combined phase 1A and 1B dataset, as well as determining the relationship between achieved RB006 concentration and elevation in aPTT. These models revealed doses of RB006≥0.75 mg/kg result in the achievement of plasma concentrations ≥15 μg/ml RB006, levels which are associated with essentially complete inhibition of plasma FIX activity, representing the maximal antithrombotic effect that can be achieved with RB006.

Phase 1C was designed to (a) affirm that RB006 administered in a weight-based fashion would lead to reliable high levels of FIX inhibition, (b) to study repeated administration of RB006, and (c) to establish the relationship between variable levels of RB006 reversal by altering the ratio of administered RB007/RB006. Based on the findings of phases IA and 1B, a weight-based dose of

0.75 mg/kg was selected to effect near complete inhibition of FIX [19].

Thirty-two healthy volunteers were randomized in a double-blind fashion to fixed doses of RB006 (0.75 mg kg^{-1}) administered on days 1, 3, and 5, followed 60 min later by varying, de-escalating doses of RB007 (repeat control agent dosing, 1.5–0.094 $mg kg^{-1}$), or double placebo [19]. As predicted by the 1A and 1B studies, administration of this weight-based dose yielded a reliable average increase in the aPTT of 241±22%, corresponding to >99% FIX inhibition [19]. No bleeding or other safety signals were observed.

Repetitive dosing resulted in highly reproducible effects on aPTT, which were consistent in each drug treatment cycle. Consistent with the results of phases 1A and 1B, RB007 administered in 2:1 ratio to RB006 resulted in normalization of aPTT to baseline, an observation that was consistent with each consecutive drug-control cycle.

Administration of variable (lower) doses of RB007 resulted in a dose-dependent graded response on degree of RB006 reversal. Dose ratios with respect to RB006 of 1:1, 0.5:1, 0.3:1, 0.2:1, and 0.125:1 resulted in reversal of approximately 100%, 84%, 74%, 51%, and 41% of RB006 anticoagulant activity, respectively, within 15 min of administration (Fig. 4) [19]. These results were used to select RB007 dosing in a phase 2A pilot study in patients undergoing elective percutaneous coronary intervention (PCI).

During PCI, monitoring of the level of antithrombotic effect using a point-of-care (POC) assay is highly desirable. To establish the usefulness of a POC whole-blood aPTT assay, the association between this and laboratory plasma aPTT was determined. A linear relationship between a point-of-care whole-blood aPTT and centrally determine aPTT was observed, with a correlation coefficient of 0.76.

Phase 2A: Reversal PCI

Phases 1A, 1B, and 1C defined a dose (≥0.75 mg/kg) of RB006 which effectively inhibited its target, FIX, in both healthy volunteers and stable patients with CAD by >99%, while also defining the relationship between administered RB007 dose and the absolute degree of RB006 reversal. These findings paved the way for the translation of the REG1 system to the care of patients in the clinical arena.

The performance of PCI mandates high levels of antithrombotic therapy to inhibit thrombus formation both on foreign bodies as well as in the arterial circulation during the procedure. However, following the PCI procedure, a reduction in anticoagulation levels is needed to enable sheath removal and subsequent hemostasis. These demands mirror the theoretical capabilities of the REG1 system; therefore, it was elected to apply the REG1 system to clinical use in the treatment of clinically stable patients undergoing elective PCI.

Phase 2A, reversal PCI (NCT00113997) [30] explored the safety and feasibility of REG1 as sole anticoagulant during elective PCI, with variable levels of reversal post-procedure to allow sheath removal. As a safety and feasibility study, the primary endpoint in reversal PCI was major in hospital bleeding and the composite of death, nonfatal myocardial infarction, and urgent target vessel revascularization through day 14. In addition, an assessment of catheter, equipment, and peri-procedural thrombosis was included as a secondary endpoint.

To minimize patient risk, the first two subjects were treated with RB006 and an intravenous glycoprotein IIb/IIIa antagonist to enhance safety by ensuring effective platelet inhibition during PCI. After safety committee review, 24 patients were randomized in a 5:1 fashion to REG1 system or unfractionated heparin.

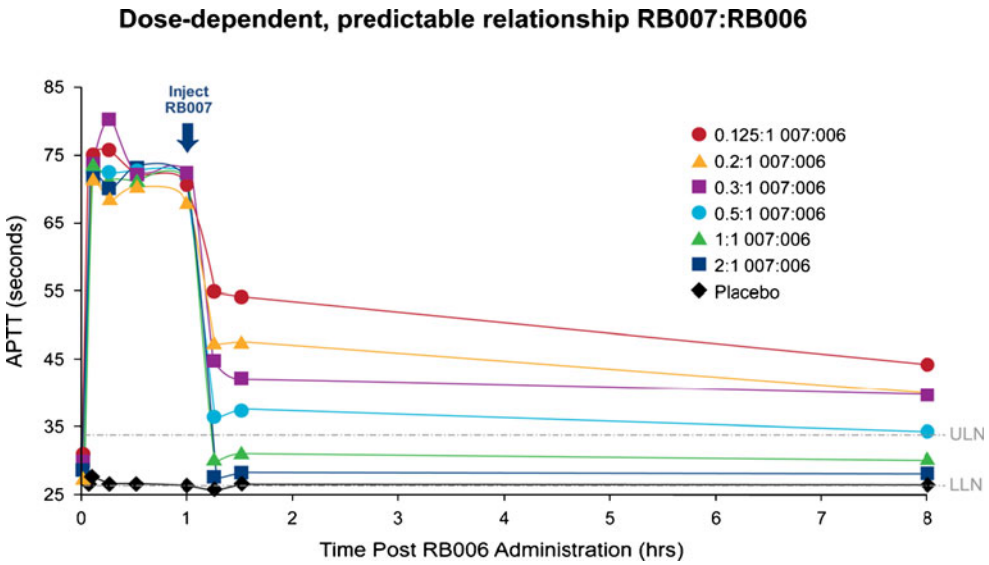

Fig. 4 Variable ratios of RB007/RB006 result in partial and titratable RB006 activity reversal

The first ten patients treated with REG1 underwent PCI followed by partial (50%) reversal of RB006 activity to mitigate the risk that full reversal post-PCI would result in thrombotic complications. After a 4-h observation period, the remaining fully reversing dose of RB007 was administered (2:1 weight-based total dose), followed by arterial sheath removal. In a second phase (n=10 REG1 patients), complete (100%) reversal of RB006 activity was effected immediately following the procedure coupled with immediate arterial sheath removal.

Key findings from this study included stable and reliable anticoagulation with a single RB006 dose as assessed prior to and after completion of the PCI procedure. At 5 min after RB006 administration and at the end of the procedure, whole-blood aPTT levels were 148.5 and 145 s, and ACT levels were 223 and 236 s, respectively, demonstrating no change in RB006 effect during the conduct of PCI. RB007 effected both partial and full reversal consistent with what was predicted based on phase 1C (Fig. 5). In addition, full reversal allowed safe immediate sheath removal [30].

RADAR: a Phase 2B Study in Acute Coronary Syndromes

The RADAR study (A Randomized, Partially-Blinded, Multi-Center, Active-Controlled, Dose-Ranging Study Assessing the Safety, Efficacy, and Pharmacodynamics of the REG1 Anticoagulation System Compared to Unfractionated Heparin or Low Molecular Heparin in Subjects with Acute Coronary Syndromes, NCT 00932100) is a randomized, partially blinded, multi-center active-controlled, dosing ranging phase 2B investigation designed to assess the safety, efficacy, and pharmacodynamic properties of the REG1 anticoagulation system in patients with ACS. Based upon the consistently high level of inhibition of FIXa activity achieved with the 1-mg/kg RB006 dose in reversal PCI, it was elected to continue to use this RB006 dose in RADAR.

RADAR will extend the clinical use of RB006 to prothrombotic patients suffering an ACS and will verify the adequacy of a 1-mg/kg dose of RB006 in this patient population to both adequately suppress FIX activity and ischemic events. Second, RADAR will determine the optimal range of RB007 mediated reversal of RB006 post-PCI to allow safe early sheath removal with mitigated bleeding.

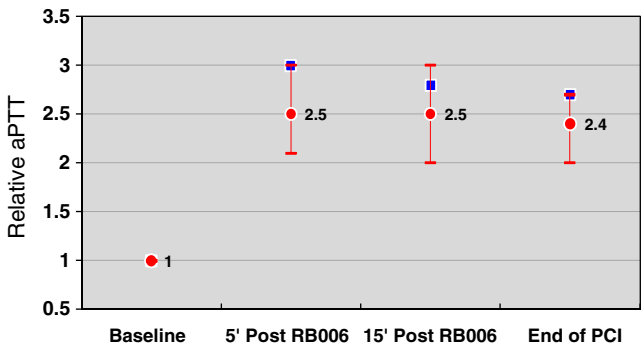

Fig. 5 RB006 (1 mg/kg) provides stable levels of anticoagulation during performance of elective PCI

A total of 800 patients with ACS undergoing cardiac catheterization will be enrolled and randomly assigned open label 3:1 to REG1 vs. heparin. REG1, patients will be randomized in a blinded fashion to one of four RB007 dose levels, designed to reverse 25%, 50%, 75%, or 100% of RB006 activity immediately after completion of cardiac catheterization (Fig. 6). Results are expected in early 2011 and will inform dose selection of both the RB006 and RB007 components for future studies.

Subcutaneous Aptamer Delivery

The development of anticoagulants for broad clinical indications, including long-term thromboprophylaxis, must consider options that are logical and attractive for both patients and practicing clinicians. Subcutaneous (SQ) drug delivery has been widely utilized to prolong systemic exposure. Administration of small and large molecules into the SC space results in different absorption profiles and therapeutic consequences of drugs. In general, absorption following SC injection is thought to be rapid and complete for most “small” drug molecules. In comparison, the absorption kinetics of large molecular mass compounds (e.g., therapeutic proteins and nucleic acids) following SC injection are typically prolonged, with delays in time to maximum concentration and prolonged terminal half-lives relative to that following IV administration. There is a linear relationship between the molecular weight (as a surrogate for molecular size) of an injected protein and the proportion of the dose absorbed from the SC injection site into the peripheral lymphatics, then to the central lymphatics before draining into the plasma compartment via the thoracic duct [31]. Near complete peripheral lymphatic absorption of compounds with a molecular weight range of ≥30–40 kDa has been observed in sheep. Given the molecular weight of RB006 (~50 kDa), the lymphatic system likely plays an important role in the absorption of RB006 from the SC injection site.

The first successful delivery of an aptamer by subcutaneous injection [32, 33] has been recently reported. RB006 administered to monkeys led to a dose-dependent prolongation of aPTT, with peak values occurring approximately 9–12 h following subcutaneous injection. In contrast to IV administration, the duration of effect based on a detectable pharmacodynamic activity was approximately 7 days. RB007, given IV, rapidly reversed RB006; however, a

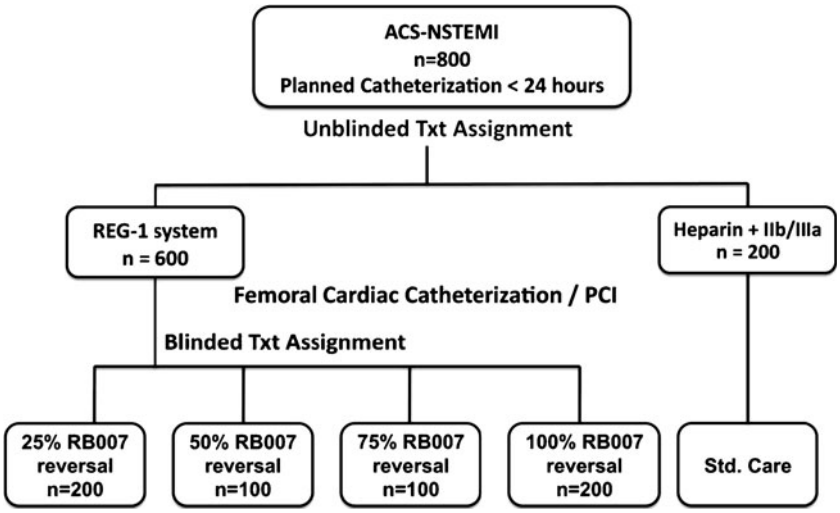

Fig. 6 RADAR study flow

return of an anticoagulant effect several hours later supports either a subcutaneous or lymphatic system depot of RB006, which is slowly released into the circulation [32, 34]. Full sustained reversal would likely require intermittent RB007 dosing to continue to reverse RB006 as it is absorbed into the plasma compartment, with frequency of additional doses and timing dependent upon the time elapsed between RB006 dosing and need for reversal.

These findings were extended to human administration in a recently completed phase I SC101 study [33]. RB006 was administered subcutaneously in a single ascending dose study involving 36 healthy subjects. Doses ranging from 0.5 to 3 mg/kg achieved dose linear RB006 plasma concentrations covering the ranges observed in prior studies of intravenous RB006 administration. In contrast to studies of intravenously delivered RB006, there was a delayed appearance in plasma (mean 2.5 h), a delayed t_{max} (mean 72 h), and a longer $t_{1/2}$ (>7 days) with subcutaneous drug delivery. These findings suggest that a single dose of RB006 administered soon after surgery or for treatment of DVT could be used to provide gradual onset of anticoagulation while maintaining adequate levels of anticoagulation for many days after a single dose. If the need for reversal arose, dosing with intravenous RB007 could be used to affect immediate reversal, with additional doses potentially required to maintain normalization of coagulation depending upon the time elapsed since RB006 administration.

The anticoagulation system consisting of SQ RB006 paired with IV RB007 is referred to as REG2. The REG2 system may also find clinical application in situations in which short-term reversal is particularly attractive. For instance in patients on chronic anticoagulation in which "bridging" to surgery is currently performed with heparin (which frequently requires hospitalization), a single SQ RB006 dose could be administered, allowing oral anticoagulant therapy to dissipate over the course of several days. At the time of surgery, a single dose of RB007 could be used to fully reverse RB006 activity, after which the patient would regain an anticoagulant effect as lymphatic stores of RB006 enter the systemic circulation.

Aptamers Targeting Thrombin

The central role played by thrombin in not only coagulation by platelet and endothelial cell activation has made this a frequent target for the development of novel antithrombotic entities [35]. The presence of multiple thrombin binding sites offers the possibility that thrombin inhibitors may have variable clinical effects, depending on the specificity of the antithrombotic for each thrombin site.

Aptamers to thrombin have been under development for the last two decades [36, 37]; however, clinical development has lagged. The initially developed aptamer was determined to bind to exosite I, responsible for thrombin's affinity for fibrinogen, factors XI, XIII, V, and VIII, as well as thrombomodulin. Archemix (Cambridge, MA, USA) has pursued the development of an antithrombin aptamer ARC183, releasing preliminary results from a phase I trial, which demonstrated a rapid dose-related anticoagulant activity, with rapid dissipation of pharmacodynamic activity after discontinuation of the drug infusion [35]. The level of drug required to achieve adequate anticoagulant activity resulted in a suboptimal dosing profile mandating a continuous infusion [35], and further development was curtailed.

The development of other thrombin aptamers is ongoing. NU172, an antithrombin aptamer, completed phase I testing [37], and a phase 1B study is planned (www.clinicaltrials.gov, NCT00808964) but not yet recruiting patients.

Furthermore, the design of more effective and efficient strategies to inhibit thrombin using aptamers with affinities for multiple thrombin binding sites has proceeded [38]. One approach has been to link an aptamer (HD1) with affinity for exosite I with a separate exosite 2 specific aptamer (HD22). The resulting bivalent aptamer (HD1–22) has demonstrated 4–10-fold higher affinities for thrombin compared with its individual components [39]. The anticoagulant properties of this bivalent aptamer have been compared with clinically available direct thrombin inhibitors, demonstrating higher thrombin inhibiting capacity, as well as greater potency to inhibit thrombin-mediated platelet aggregation compared with bivalirudin (2-fold more potent) or argatroban (5-fold more potent). In addition, the activity of HD1–22 was fully reversible upon addition of several antidote oligonucleotides.

Nimjee and colleagues have used an alternative approach, hypothesizing that administration of an aptamer "cocktail" containing sequences specific for each exosite may provide complementary effects. Based on crystal structures of the aptamers ARC183 and TOG25 bound to thrombin [40–43], they postulated that binding of the two oligonucleotides to thrombin would be independent—a hypothesis that was subsequently verified based on binding affinities of each aptamer alone and in combination.

Pharmacodynamic evaluation demonstrated that the two aptamers displayed disparate and synergistic effects on coagulation assays [44]. For instance, the exosite-1 binding DNA aptamer ARC183 prolonged the aPTT 4.4-fold, while the exosite-2 binding RNA aptamer TOG25 prolonged the aPTT 3.2-fold. When both aptamers were administered, the aPTT increased 8.6-fold [44]. Similar effects were observed on the prothrombin time. Effects on thrombin clot time, which is a reflection of thrombin catalytic activity, followed a different pattern, with a pronounced effect observed in the presence of ARC183 and, in contrast, minimal effects with TOG25 (although the presence of TOG25 decreased the concentration of ARC183 required to produce maximal effects). Platelet activation by thrombin was also affected by each aptamer but was synergistically inhibited by their combination [44]. These studies not only demonstrate the feasibility and attractiveness of inhibiting both thrombin binding sites to maximize effectiveness but also help to define the contribution of each in determining thrombin activity.

Aptamers Targeting Prothrombinase

The ability of aptamers to target binding motifs that have proven difficult to target with peptide/antibody inhibitors is highlighted both by the success of RB006 to affect FIX inhibition, which has not successful been targeted by other motifs, as well as the recent description of an aptamer Xa inhibitor which effectively inhibits formation of the Xa–Va complex, possibly via allosteric effects that alter Xa conformation.

Platelet Targets for Aptamer Development

The successful development of an aptamer-based anticoagulant has led to interest in expansion of aptamer targets to other thrombotic mediators including platelets. Currently available antiplatelet therapies include inhibitors of cyclooxygenase-1-mediated thromboxane synthesis (aspirin) as well as the G-protein coupled P2Y12 ADP receptor (ticlopidine, clopidogrel, prasugrel), which are generally amenable to small molecule therapeutics. In contrast, the experience with oral inhibitors of targets whose primary activity is mediated by protein–protein interaction, such as the integrin glycoprotein IIb/IIIa receptor was quite disappointing. Successful inhibition of such receptors has typically required non-traditional therapeutic modalities, such as monoclonal antibodies and peptidomimetics. As a class, aptamers have shown a robust ability to block protein–protein interactions and therefore may open up potential antiplatelet targets beyond the traditional GPCRs to those whose activity is mediated by direct protein–protein interactions. Aptamers to such targets would offer unprecedented control of antiplatelet therapy.

von Willebrand Factor

The bleeding phenotype inherent to patients with von Willebrand factor (vWF) disease point to the central role this multimeric plasma protein plays in normal hemostasis [45]. vWF is an attractive target for therapeutic application for a variety of reasons. vWF plays a central role in recruitment and activation of platelets at sites of vascular injury via interaction with the GPIb-IX-V as well as IIb/IIIa receptors. Second, the vWF–platelet interaction acts to bind and activate platelets independent of other pathways commonly targeted in clinical practice, including the cyclooxygenase pathway and the P2Y12 ADP receptor pathways. Third, the vWF–GPIb-IX-V interaction plays a vital role in thrombus generation after endothelial disruption, as occurs commonly during acute thrombotic events. Last, vWF is activated under conditions of high shear stress; thus, this pathway is particularly important in the arterial circulation.

Inhibition of the vWF–GPIb-IX-V interaction decreases arterial thrombosis in animal models; however, as patients with von Willebrand's disease are particularly prone to bleeding at sites of vascular injury, as is iatrogenically

incurred during procedural vascular access or surgery, mimicking this disease via inhibition of this interaction could provoke bleeding unless the intensity of therapy is readily controllable. The use of aptamers to inhibit vWF offers one approach to effectively inhibit this important interaction while allowing restoration of normal hemostasis by administration of controlling agent when required.

The complexities of vWF synthesis and activity further suggest that depending on aptamer binding site, variable effects on antithrombotic efficacy and propensity for bleeding might be expected, much as there are highly variable phenotypes associated with defects in vWF synthesis and assembly [45–48]. Oney and colleagues have generated a series of aptamer clones which bind to vWF domains in variable combinations [49]. Although showing similar affinity for binding to vWF, platelet inhibition as measured using a PFA-100 assay varied according to the domain binding site. Two clones, clone 9.3 and 9.14, were found to specifically inhibit vWF–GPIb-IX-V interaction resulting in effective platelet inhibition. An oligonucleotide controlling agent, A06, actively reversed aptamer 9.14 binding to vWF, with resulting reversal of 9.14's anti-platelet effects as determined using a PFA-100 assay [49]. Thus, this VWF aptamer represents the first reported example of an aptamer-control agent pair.

Clinical development of a therapeutic aptamer targeting the vWF–GPIb interaction (ARC1779) is ongoing. A phase I dose escalation study [50] demonstrated that infusion of ARC1779 was well tolerated. Pharmacokinetic analysis demonstrated that peak plasma concentrations were rapidly achieved, distribution was limited to the vascular space, and that the agent had a half-life of approximately 2 h.

vWF inhibition, as measured by the amount of free vWF in the plasma, was dose dependent and approached >97% with the highest dose (1.0 mg/kg). The extent of inhibition could be titrated based on dose, with recovery of vWF activity of 10% within 8 h and return to baseline by 12 to 16 h. At doses of >0.3 mg/kg, platelet inhibition as measured by prolongation PFA-100 reached maximal levels, with recovery of platelet function closely mirroring the recovery of vWF activity. Cutaneous bleeding times (CBT), which increased after ARC1779 administration to maximal values, showed faster recovery, with CBT partially normalizing at 4 h while vWF activity and platelet reactivity were still fully inhibited. There were no bleeding events in the study, demonstrating that transient high intensity vWF inhibition may be safe, at least in a non-surgical setting.

These findings have led to the conduct of a phase 2a randomized, double-blind placebo-controlled clinical trial of ARC1779 exploring the use of ARC1779 in the setting of surgical carotid endarterectomy; however, this trial has recently been terminated due to slow enrollment (www.clinicaltrials.gov, NCT00742612). ARC1779 is also under clinical development for the treatment of patients with thrombotic thrombocytopenia purpura [51, 52], for which it has received an "orphan drug" designation from both the FDA and EMA.

Glycoprotein VI Receptor

Glycoprotein VI (GPVI) is the major platelet collagen receptor and is required for collagen-mediated platelet activation and firm adhesion of platelets to a damaged vessel wall. GPVI is a 62–65-kDa glycoprotein of the immunoglobulin superfamily, consisting of two Ig C2 loops that contain a collagen binding domain, a mucin-like core region, a membrane spanning domain, and an intracellular domain. Expression of GPVI is specific to platelets [53]. On the platelet surface, GPVI is a functional dimer associated with the FcR γ-chain co-receptor. The interaction of GPVI with collagen is one of the most potent activators of platelets, triggering both inside-out activation of the platelet integrins $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ as well as secretion of platelet granule contents (which in turn activates nearby circulating platelets). GPVI is specific for the fibrillar vascular collagens, types I, II, III, and IV [54]. Signaling via GPVI activation may be mediated indirectly through the γ-chain of FcR or directly through the through the GPVI cytoplasmic domain. Congenital or acquired abnormalities of the GPVI receptor are extremely rare with only 11 case reports to date. This phenotype is manifested by a variable spectrum of mild to severe bleeding episodes [55].

GPVI is expressed at low to moderate levels on platelets, with a typical receptor number of ~1,200 per platelet [53]. However, increased levels of GPVI expression leads to platelets hyper-reactive to collagen and thus predispose individuals to a disease, or directly cause a disease state mediated by the platelet–collagen interaction. Overexpression of GPVI has been linked to the onset of thrombotic vascular diseases, including transient ischemic attacks and acute coronary syndromes [56–58]. Additionally, diabetes is associated with enhanced collagen-mediated platelet activation, and GPVI expression is significantly higher in individuals with diabetes as compared to those without [59]. Mice deficient in GPVI are resistant to thrombosis when the primary insult in the model exposes the major vascular type collagens I and III [60]. Further, radiolabeled GPVI specifically images sites of vascular lesions in mice as it binds specifically to the injured region, indicating that collagen is exposed at these sites [61]. These findings have been translated to humans, where it has been shown recently that human atheromatous plaques from patients with carotid stenosis contained collagen type I and type III

structures that were able to activate platelets [62]. Together, these epidemiologic findings in human and molecular genetic studies in mice indicate GPVI is a potentially attractive target for antiplatelet therapies.

Accordingly, the future role of the GPVI receptor as a target for clinical therapy is the focus of active investigation. Peptides, mAbs and Fab fragments that bind to GPVI and inhibit platelet activation, adhesion, and aggregation have been described (e.g., [62–65]). The development of the mAbs has been limited by the development of mAb-mediated severe thrombocytopenia [63]. Testing of anti-GPVI Fab fragments has advanced to primate models [64]. In addition to the antibody and antibody fragment approaches, dimeric forms of soluble GPVI have also been shown to block the GPVI–collagen interaction and prevent thrombus formation in animal models [65]. In general, all the agents described have yielded positive results in animal models of thrombosis, including models mimicking ACS as well as stroke. In these models, no increase in bleeding has been observed, suggesting that active regulation of these agents may not be as essential; however, it is not known to what degree inhibition of GPVI in humans may result in bleeding tendencies, and should bleeding arise, the availability of reversal agents remains clinically attractive. Actively controllable aptamer inhibitors of GPVI are under development, with initial human testing anticipated in 2011 (C. Rusconi, personal communication).

Aptamers to this and other platelet targets would offer new avenues of platelet inhibition and unprecedented control of antiplatelet therapy. Current used therapies (aspirin, ticlopidine, prasugrel, and clopidogrel) result in irreversible platelet inhibition, requiring either the production of new platelets or platelet transfusion to restore normal platelet function. While newer therapies in clinical development result in somewhat abbreviated and reversible platelet inhibition (e.g., ticagrelor), daily or twice daily administration will be required to achieve and maintain a consistent intensity of platelet inhibition. The use of a long-acting aptamer platelet antagonist would allow infrequent dosing followed by reversal should bleeding or the need for invasive procedures arise.

Conclusions

Nucleic acid aptamers, by encoding for their own controlling agents or by being controllable by nucleic acid binding polymers, introduce a translatable platform for drug development, unprecedented in cardiovascular disease. As reversible and highly regulatable agents, they may represent an ideal strategy for the management of thrombotic disorders and conditions requiring either short-term or graded antithrombotic therapy. REG1, a specific FIXa inhibitor and its active controlling agent, has undergone extensive preclinical and early phase clinical testing and is a leading clinical candidate currently in phase 2B investigation in ACS. REG2, a subcutaneous formulation of RB006 with IV active controlling agent, is in phase 1 development. Development programs of aptamer antithrombotics targeting thrombin and vWF are ongoing, while factor Xa, prothrombin, platelet GPVI, and multi-site aptamers are in preclinical development. Each of these programs is unique and highlights properties specific to aptamers, including the ability to target binding sites that have proven resistant to peptide/antibody inhibition. As highly specific and readily controllable drugs, aptamers translate well to a patient disease and/or clinical condition-specific approach to antithrombotic therapy in cardiovascular disease.

Conflicts of Interest Dr. Rusconi is a founder, employee, and stockholder in REGADO Biosciences; Dr. Zelenkofske is an employee and stockholder in REGADO Biosciences. Dr. Sullenger is a founder and stockholder in REGADO Biosciences; Drs. Povsic and Becker are employed by the DCRI, which has received research funding from REGADO Biosciences.

References

1. Harrington, R. A., Becker, R. C., Cannon, C. P., Gutterman, D., Lincoff, A. M., Popma, J. J., et al. (2008). Antithrombotic therapy for non-ST-segment elevation acute coronary syndromes*. *Chest, 133*, 670S–707S. doi:10.1378/chest.08-0691.
2. Wiviott, S. D., Braunwald, E., McCabe, C. H., Montalescot, G., Ruzyllo, W., Gottlieb, S., et al. (2007). Prasugrel versus clopidogrel in patients with acute coronary syndromes. *The New England Journal of Medicine, 357*, 2001–2015. doi:10.1056/NEJMoa0706482.
3. Stone, G. W., Ware, J. H., Bertrand, M. E., Lincoff, A. M., Moses, J. W., Ohman, E. M., et al. (2007). Antithrombotic strategies in patients with acute coronary syndromes undergoing early invasive management: one-year results from the acuity trial. *JAMA, 298*, 2497–2506. doi:10.1001/jama.298.21.2497.
4. Becker, R. C., Moliterno, D. J., Jennings, L. K., Pieper, K. S., Pei, J., Niederman, A., et al. (2009). Safety and tolerability of SCH 530348 in patients undergoing non-urgent percutaneous coronary intervention: a randomised, double-blind, placebo-controlled phase II study. *Lancet, 373*, 919–928.
5. Tuerk, C., & Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage t4 DNA polymerase. *Science, 249*, 505–510.
6. Ellington, A. D., & Szostak, J. W. (1990). In vitro selection of RNA molecules that bind specific ligands. *Nature, 346*, 818–822.
7. Dausse, E., Cazenave, C., Rayner, B., & Toulme, J. J. (2005). In vitro selection procedures for identifying DNA and RNA aptamers targeted to nucleic acids and proteins. *Methods in Molecular Biology, 288*, 391–410.
8. Rimmele, M. (2003). Nucleic acid aptamers as tools and drugs: Recent developments. *Chembiochem, 4*, 963–971. doi:10.1002/cbic.200300648.

9. Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H., & Eckstein, F. (1991). Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. *Science, 253*, 314–317.
10. Beigelman, L., McSwiggen, J. A., Draper, K. G., Gonzalez, C., Jensen, K., Karpeisky, A. M., et al. (1995). Chemical modification of hammerhead ribozymes. Catalytic activity and nuclease resistance. *The Journal of Biological Chemistry, 270*, 25702–25708.
11. Nimjee, S. M., Rusconi, C. P., & Sullenger, B. A. (2005). Aptamers: An emerging class of therapeutics. *Annual Review of Medicine, 56*, 555–583. doi:10.1146/annurev.med.56.062904.144915.
12. Bouchard, P. R., Hutabarat, R. M., & Thompson, K. M. (2010). Discovery and development of therapeutic aptamers. *Annual Review of Pharmacology and Toxicology, 50*, 237–257. doi:10.1146/annurev.pharmtox.010909.105547.
13. Rusconi, C. P., Yeh, A., Lyerly, H. K., Lawson, J. H., & Sullenger, B. A. (2000). Blocking the initiation of coagulation by RNA aptamers to factor VIIa. *Thromb Haemost, 84*, 841–848.
14. Doudna, J. A., Cech, T. R., & Sullenger, B. A. (1995). Selection of an RNA molecule that mimics a major autoantigenic epitope of human insulin receptor. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 2355–2359.
15. Lee, S. W., & Sullenger, B. A. (1997). Isolation of a nuclease-resistant decoy RNA that can protect human acetylcholine receptors from myasthenic antibodies. *Nature Biotechnology, 15*, 41–45. doi:10.1038/nbt0197-41.
16. Rusconi, C. P., Scardino, E., Layzer, J., Pitoc, G. A., Ortel, T. L., Monroe, D., et al. (2002). RNA aptamers as reversible antagonists of coagulation factor IXa. *Nature, 419*, 90–94.
17. Rusconi, C. P., Roberts, J. D., Pitoc, G. A., Nimjee, S. M., White, R. R., Quick, G., et al. (2004). Antidote-mediated control of an anticoagulant aptamer in vivo. *Nature Biotechnology, 22*, 1423–1428.
18. Nimjee, S. M., Rusconi, C. P., Harrington, R. A., & Sullenger, B. A. (2005). The potential of aptamers as anticoagulants. *Trends in Cardiovascular Medicine, 15*, 41–45.
19. Chan, M. Y., Rusconi, C. P., Alexander, J. H., Tonkens, R. M., Harrington, R. A., & Becker, R. C. (2008). A randomized, repeat-dose, pharmacodynamic and safety study of an antidote-controlled factor IXa inhibitor. *Journal of Thrombosis and Haemostasis, 6*, 789–796.
20. Oney, S., Lam, R. T., Bompiani, K. M., Blake, C. M., Quick, G., Heidel, J. D., et al. (2009). Development of universal antidotes to control aptamer activity. *Nature Medicine, 15*, 1224–1228.
21. Hoffman, M., Monroe, D. M., Oliver, J. A., & Roberts, H. R. (1995). Factors IXa and Xa play distinct roles in tissue factor-dependent initiation of coagulation. *Blood, 86*, 1794–1801.
22. Monroe, D. M., Hoffman, M., & Roberts, H. R. (1996). Transmission of a procoagulant signal from tissue factor-bearing cells to platelets. *Bood Coagulation and Fibrinolysis, 7*, 459–464.
23. Howard, E. L., Becker, K. C. D., Rusconi, C. P., & Becker, R. C. (2007). Factor IXa inhibitors as novel anticoagulants. *Arteriosclerosis, Thrombosis, and Vascular Biology, 27*, 722–727. doi:10.1161/01.ATV.0000259363.91070.f1.
24. Sramek, A., Kriek, M., & Rosendaal, F. (2003). Decreased mortality of ischaemic heart disease among carriers of haemophilia. *Blood, 108*, 52–66.
25. Tuinenburg, A., Mauser-Bunschoten, E., Verhaar, M., Biesma, D., & Schutgens, R. (2009). Cardiovascular disease in patients with hemophilia. *Journal of Thrombosis and Haemostasis, 7*, 247–254.
26. Bolton-Maggs, P. H. B., & Pasi, K. J. (2003). Haemophilias a and b. *Lancet, 361*, 1801–1809.
27. Dyke, C. K., Steinhubl, S. R., Kleiman, N. S., Cannon, R. O., Aberle, L. G., Lin, M., et al. (2006). First-in-human experience of an antidote-controlled anticoagulant using RNA aptamer technology: A phase 1a pharmacodynamic evaluation of a drug–antidote pair for the controlled regulation of factor IXa activity. *Circulation, 114*, 2490–2497. doi:10.1161/circulationaha.106.668434.
28. Nimjee, S. M., Keys, J. R., Pitoc, G. A., Quick, G., Rusconi, C. P., & Sullenger, B. A. (2006). A novel antidote-controlled anticoagulant reduces thrombin generation and inflammation and improves cardiac function in cardiopulmonary bypass surgery. *Molecular Therapy, 14*, 408–415.
29. Chan, M. Y., Cohen, M. G., Dyke, C. K., Myles, S. K., Aberle, L. G., Lin, M., et al. (2008). Phase 1b randomized study of antidote-controlled modulation of factor IXa activity in patients with stable coronary artery disease. *Circulation, 117*, 2865–2874. doi:10.1161/circulationaha.107.745687.
30. Cohen, M. G., Purdy, D. A., Rossi, J. S., Grinfeld, L. R., Myles, S. K., Aberle, L. G., et al. (2010). First clinical application of an actively reversible direct factor IXa inhibitor as an anticoagulation strategy in patients undergoing percutaneous intervention. *Circulation, 122*, 614–622.
31. McLennan, D., Porter, C., Edwards, G., Martin, S., Heatherington, A., & Charman, S. (2005). Lymphatic absorption is the primary contributor to the systemic availability of epoetin alfa following subcutaneous administration to sheep. *The Journal of Pharmacology and Experimental Therapeutics, 313*, 345–351.
32. Rusconi, C. P., Kornbrust, D., & Zelenkofske, S. L. (2010). Subcutaneous FIXa inhibitor provides prolonged anticoagulation: the first successful subcutaneous application of an aptamer. *Journal of the American College of Cardiology, 55*, A172.E1618.
33. Zelenkofske, S., Rusconi, C. P., Damiento, C., Wargin, W., Medlock, M., Becker, R.C. (2010) *Subcutaneous rb006: A direct FIXa inhibitor provides prolonged anticoagulation with rapid reversal: The first clinical experience with the reg2 system*. Atherothrombosis and Vascular Biology Meeting, San Francisco, CA, April 5, 2010.
34. Eikelboom, J. W., Zelenkofske, S. L., & Rusconi, C. P. (2010). Coagulation factor IXa as a target for treatment and prophylaxis of venous thromboembolism. *Arteriosclerosis, Thrombosis, and Vascular Biology, 30*, 382–387. doi:10.1161/atvbaha.110.203117.
35. Schwienhorst, A. (2006). Direct thrombin inhibitors—a survey of recent developments. *Cellular and Molecular Life Sciences, 63*, 2773–2791.
36. Bock, L., Griffin, L., Latham, J., Vermaas, E., & Toole, J. (1992). Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature, 355*, 564–566.
37. DeAnda, A. J., Coutre, S., Moon, M., Vial, C., Griffin, L., Law, V., et al. (1994). Pilot study of the efficacy of a thrombin inhibitor for use during cardiopulmonary bypass. *The Annals of Thoracic Surgery, 58*, 344–350.
38. Buff, M. C. R., Schafer, F., Wulffen, B., Muller, J., Potzsch, B., Heckel, A., et al. (2010). Dependence of aptamer activity on opposed terminal extensions: Improvement of light-regulation efficiency. *Nucleic Acids Research, 38*, 2111–2118. doi:10.1093/nar/gkp1148.
39. Muller, J., Wulffen, B., Potzsch, B., & Mayer, G. (2007). Multidomain targeting generates a high-affinity thrombin-inhibiting bivalent aptamer. *Chembiochem, 8*, 2223–2226.
40. Jeter, M. L., Ly, L. V., Fortenberry, Y. M., Whinna, H. C., White, R. R., Rusconi, C. P., et al. (2004). RNA aptamer to thrombin binds anion-binding exosite-2 and alters protease inhibition by heparin-binding serpins. *FEBS Letters, 568*, 10–14.
41. Long, S. B., Long, M. B., White, R. R., & Sullenger, B. A. (2008). Crystal structure of an RNA aptamer bound to thrombin. *RNA, 14*, 2504–2525.
42. Padmanabhan, K., Padmanabhan, K. P., Ferrara, J. D., Sadler, J. E., & Tulinsky, A. (1993). The structure of alpha-thrombin inhibited by a 15-mer single-stranded DNA aptamer. *The Journal of Biological Chemistry, 268*, 17651–17654.

43. Padmanabhan, K., & Tulinsky, A. (1996). An ambiguous structure of a DNA 15-mer thrombin complex. *Acta Crystallographica. Section D: Biological Crystallography, 52*, 272–282.
44. Nimjee, S. M., Oney, S., Volovyk, Z., Bompiani, K. M., Long, S. B., Hoffman, M., et al. (2009). Synergistic effect of aptamers that inhibit exosites 1 and 2 on thrombin. *RNA, 15*, 2105–2111.
45. De Meyer, S. F., Deckmyn, H., & Vanhoorelbeke, K. (2009). von Willebrand factor to the rescue. *Blood, 113*, 5049–5057. doi:10.1182/blood-2008-10-165621.
46. Sadler, J. (2005). New concepts in von Willebrand disease. *Annual Review of Medicine, 56*, 173–191.
47. Sadler, J. (2005). von Willebrand factor: Two sides of a coin. *Journal of Thrombosis and Haemostasis, 3*, 1702–1709.
48. Ruggeri, Z. M. (2003). von Willebrand factor, platelets and endothelial cell interactions. *Journal of Thrombosis and Jaemostasis, 1*, 1335–1342.
49. Oney, S., Nimjee, S. M., Layzer, J., Que-Gewirth, N., Ginsburg, D., Becker, R. C., et al. (2007). Antidote-controlled platelet inhibition targeting von Willebrand factor with aptamers. *Oligonucleotides, 17*, 265–274. doi:10.1089/oli.2007.0089.
50. Gilbert, J. C., DeFeo-Fraulini, T., Hutabarat, R. M., Horvath, C. J., Merlino, P. G., Marsh, H. N., et al. (2007). First-in-human evaluation of anti von Willebrand factor therapeutic aptamer ARC1779 in healthy volunteers. *Circulation, 116*, 2678–2686. doi:10.1161/circulationaha.107.724864.
51. Firbas, Cs., Jilma, B., Wagner, P. G., Hutabarat, R., Schaub, R. G., Gilbert, J. C., Knoebl, P. (2008) *Anti von Willebrand factor aptamer arc 1779 for refractory thrombotic thrombocytopenic purpura*. ASH Annual Meeting Abstracts 112, p. 2298
52. Ima, B. J., Jilma, P., Paulinska, P., Gilbert, J. C., Hutabarat, R., Wagner, T., Knoebl, P. (2008) *Proof of concept for the anti von Willebrand factor aptamer in patients with relapsing thrombotic thrombocytopenic purpura (TTP)*. ASH Annual Meeting Abstracts 112, p. 2290
53. Jandrot-Perrus, M., Busfield, S., Lagrue, A.-H., Xiong, X., Debili, N., Chickering, T., et al. (2000). Cloning, characterization, and functional studies of human and mouse glycoprotein VI: A platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood, 96*, 1798–1807.
54. Jung, S. M., Takemura, Y., Imamura, Y., Hayashi, T., Adachi, E., & Moroi, M. (2008). Collagen-type specificity of glycoprotein VI as a determinant of platelet adhesion. *Platelets, 19*, 32–42.
55. Arthur, J. F., Dunkley, S., & Andrews, R. K. (2007). Platelet glycoprotein VI-related clinical defects. *British Journal Haematology, 139*, 363–372.
56. Bigalke, B., Haap, M., Stellos, K., Geisler, T., Seizer, P., Kremmer, E., et al. (2010). Platelet glycoprotein VI (GPVI) for early identification of acute coronary syndrome in patients with chest pain. *Thrombosis Research, 125*, e184–e189.
57. Bigalke B, Stellos K, Geisler T, Kremmer E, Seizer P, May AE, Lindemann S, Gawaz M (2009) Glycoprotein VI for diagnosis of acute coronary syndrome when ECG is ambiguous. *Int J Cardiol*. doi:10.1016/j.ijcard.2009.12.026
58. Bigalke, B., Stellos, K., Geisler, T., Lindemann, S., May, A., & Gawaz, M. (2010). Glycoprotein VI as a prognostic biomarker for cardiovascular death in patients with symptomatic coronary artery disease. *Clinical Research in Cardiology, 99*, 227–233.
59. Cabeza, N., Li, Z., Schulz, C., Kremmer, E., Massberg, S., Bultmann, A., et al. (2004). Surface expression of collagen receptor Fc receptor-γ/glycoprotein VI is enhanced on platelets in type 2 diabetes and mediates release of CD40 ligand and activation of endothelial cells. *Diabetes, 53*, 2117–2121. doi:10.2337/diabetes.53.8.2117.
60. Denis, C. V., & Wagner, D. D. (2007). Platelet adhesion receptors and their ligands in mouse models of thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology, 27*, 728–739. doi:10.1161/01.atv.0000259359.52265.62.
61. Gawaz, M., Konrad, I., Hauser, A. I., Sauer, S., Li, Z., Wester, J.-J., et al. (2005). Non-invasive imaging of blycoprotein VI binding to injured arterial lesions. *Thrombosis and Haemostasis, 93*, 910–913.
62. Penz, S., Reininger, A. J., Brandl, R., Goyal, P., Rabie, T., Bernlochner, I., et al. (2005). Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. *The FASEB Journal, 19*, 898–909. doi:10.1096/fj.04-2748com.
63. De Meyer, S. F., Vanhoorelbeke, K., Ulrichts, H., Staelens, S., Feys, H., Salles, I., et al. (2006). Development of monoclonal antibodies that inhibit platelet adhesion or aggregation as potential anti-thrombotic drugs. *Cardiovascular and Haematologic Disorders-Drug Targets, 6*, 191–207.
64. Ohlmann, P., Hechler, B., Ravanat, C., Loyau, S., Herrenschmidt, N., Wanert, F., et al. (2008). Ex vivo inhibition of thrombus formation by an anti-glycoprotein VI Fab fragment in non-human primates without modification of glycoprotein VI expression. *Thrombosis and Haemostasis, 6*, 1003–1011.
65. Massberg, S., Konrad, I., Bultmann, A., Schulz, C., Munch, G., Peluso, M., et al. (2004). Soluble glycoprotein VI dimer inhibits platelet adhesion and aggregation to the injured vessel wall in vivo. *FASEB J., 18*, 397–399. doi:10.1096/fj.03-0464fje.