Factor IXa Inhibitors as Novel Anticoagulants

Emily L. Howard, Kristian C.D. Becker, Christopher P. Rusconi, Richard C. Becker

Abstract—Currently available anticoagulants are limited by modest therapeutic benefits, narrow clinical applications, increased bleeding risk, and drug-induced thrombophilia. Because factor IX plays a pivotal role in tissue factor (TF)–mediated thrombin generation, it may represent a promising target for drug development. Several methods of attenuating factor IX activity, including monoclonal antibodies, synthetic active site-blocked competitive inhibitors, oral inhibitors, and RNA aptamers, have undergone investigation. This review summarizes present knowledge of factor IX inhibitors with emphasis on biology, pharmacology, preclinical data, and early-phase clinical experience in humans. (Arterioscler Thromb Vasc Biol. 2007;27:722-727.)

Key Words: anticoagulants ■ coagulation ■ factor IX (fIX), monoclonal antibodies (mAb), RNA aptamers

Coagulation is a complex process wherein circulating cells and coagulation factors interface with tissue-based proteins to form an insoluble clot at sites of vascular injury. Although this dynamic process represents an advantageous response after localized vessel trauma, clot formation may also be undesirable. For example, thrombosis within the coronary or cerebrovascular beds is the proximate cause of myocardial infarction and ischemic stroke, respectively. Moreover, common procedures such as percutaneous coronary intervention, hemodialysis, blood pheresis, cardiac valve replacement, and extracorporeal circulatory support systems incite coagulation. Accordingly, the development of pharmacological agents that attenuate clot formation safely and effectively is an attractive goal for clinicians and the pharmaceutical industry. Here, we summarize the current status of factor IXa inhibitors as an emerging class of novel anticoagulants.

Cell-Based Model of Coagulation

Coagulation in vivo is best characterized as a coordinated series of cell-based events with 3 distinct phases: initiation, priming, and propagation (Figure 1).1-2 Coagulation is initiated when tissue factor (TF) binds activated factor VII, a circulating coagulation factor. In general, blood is not exposed to TF, a transmembrane protein constitutively expressed on extravascular cells. However, vascular injury exposes these extravascular TF-bearing cells to blood, and thus initiates the coagulation process. In various inflammatory states, TF expression can also be upregulated on monocytes and endothelial cells by bacterial antigens,3 inflammatory cytokines,4 and tumor necrosis factor.5

The TF/VIIa complex activates factors IX and X.6 Factor IXa is relatively stable in plasma and diffuses toward activated platelets. In contrast, factor Xa is unstable in plasma and is rapidly inhibited by TF pathway inhibitor and antithrombin III.7-8 On the surface of TF-bearing cells, factor Xa binds factor Va.9 In turn, the Xa/Va complex generates a small but sufficient amount of thrombin to cause platelet activation.10,11

In the priming phase, platelets and coagulation factors are activated by thrombin.1 Thrombin binds and cleaves platelet protease-activated receptors (PAR1 and PAR4), triggering a signaling cascade that catalyzes platelet activation and release of factor V from platelet α granules. In addition, thrombin activates factors V, VIII, and XI.

Thrombin generation is maximized on the surface of platelets during the propagation phase. The primed, activated platelets bind the IXa/VIIa “tenase” complex. Additional IXa is generated by factor Xa on the platelet surface.12 The IXa/VIIa complex, in physical proximity to Va, recruits
factor X to the platelet surface for activation. The Xa/Va complex on the platelet surface is protected from TF pathway inhibitor and antithrombin III.13,14 Enzymology studies have shown that activation of factor X by IXa/VIIIa is nearly 50/H11003 more efficient than activation by factor VIIa/TF. 15 The platelet Xa/Va complex generates a "burst" of thrombin, resulting in a stable fibrin–platelet clot.

The cell-based model of coagulation highlights the importance of the IXa/VIIIa complex in clot formation. Thus, factor IX represents an excellent target for anticoagulant therapy.

**Factor IX Structure–Function Relationships**

Factor IX is synthesized in the liver, where it undergoes vitamin K–dependent carboxylation of glutamate residues. Factor IX circulates in the plasma as a single chain zymogen with 6 domains: the amino terminal Gla domain containing 12 γ-carboxy glutamic acid residues (residues 1-40), a hydrophobic region (residues 41-46), 2 epidermal growth factor (EGF)–like domains (EGF-1 residues 47-84 and EGF-2 residues 85-127), an activation peptide (residues 146-180), and a carboxy terminal serine protease domain (residues 181-415; Figure 2).16 The protease is activated by cleavage of peptide bonds following Arg145 and Arg180, releasing the activation peptide.

The N-terminal light chain of factor IXa contains the Gla domain, which is essential for cellular membrane binding. On calcium binding, the Gla residues turn "inward," exposing a hydrophobic patch that inserts into the membrane lipid bilayer, anchoring factor IX to the cell surface.17 The 2 EGF-like domains of factor IX position the catalytic domain above the cell surface.e,18,19 Disruption of the EGF domains results in reduced binding of factor VIIa-TF and factor VIIIa.20,21

The heavy chain or catalytic domain of factor IXa consists of a substrate binding groove surrounded by 6 surface loops.18 These surface loops are important for structural integrity and interactions with factor VIIIa.16 In the absence of factor VIIIa, the active site of factor IXa has surprisingly low activity toward substrates compared with other coagulation enzymes. Association with factor VIIIa provokes a conformational change in the active site of factor IXa, triggering high enzyme activity.19

Factor IXa is inactivated by multiple factors including antithrombin III, nexin–2/amyloid β protein precursor,22 neutrophil elastase,23 and protein Z–dependent protease inhibitor.24 However, because of its incompletely formed active site in the absence of factor VIIIa, inactivation of factor IXa by these plasma factors is relatively inefficient compared with inactivation of other coagulation factors, such as Xa and thrombin. Thus, factor IXa is unique in its ability to diffuse efficiently from TF-bearing cells to platelets, and thereby serve as the critical link between the initiation and propagation phases of the coagulation reaction. Clearance of factor IXa from the circulation is mediated by low-density lipoprotein receptor–related protein, an endocytic receptor on hepatocytes.25

**Active Site-Blocked Competitive Antagonists**

**Mechanism of Action**

The earliest investigation of factor IXa inhibitors used the competitive antagonist IXai. The active site of IXa is blocked by incubation with dansyl-glutamyl-glycyl-arginyl-chloromethylketone, yielding a protein without functional coagulant activity.26 Thus, IXai functions as a competitive inhibitor of factor IXa binding to platelets.27

**Preclinical Data**

In several animal models, IXai functioned as an effective anticoagulant with limited bleeding complications. Intravenous infusion of IXai prevented thrombosis in a canine model of coronary thrombosis in a dose-dependent fashion. Animals treated with factor IXai exhibited reduced bleeding compared with animals treated with unfractionated heparin.28 A rabbit model of synthetic patch angioplasty showed that IXai resulted in effective anticoagulation with limited bleeding from puncture sites produced in synthetic vascular grafts.29 IXai also inhibited thrombus formation in a rabbit model of arterial thrombosis. In this model, bovine IXai appeared to be less effective than active site-blocked human Xa (Xai).30
However, without data regarding cross-reactivity, it is unclear how human IXai would compare to human Xai in different species.

In a canine model of cardiopulmonary bypass, IXai was associated with effective anticoagulation and limited fibrin formation in the extracorporeal circuit, as well as diminished blood loss compared with animals treated with unfractionated heparin.\(^31\) Similar results were observed in a primate model of cardiopulmonary bypass.\(^32\)

Factor IXai also exhibited anticoagulant effects in a rat model of stroke.\(^33\) Platelet and fibrin deposition after occlusion of the middle cerebral artery was markedly reduced after IXai administration. In addition, intracerebral hemorrhage occurred with decreased frequency in animals treated with factor IXai compared with animals treated with either tissue plasminogen activator or unfractionated heparin. Of particular interest, factor IXai was protective even after the onset of stroke, suggesting that microvascular thrombosis continues after primary occlusion of a major cerebral vessel and may be attenuable with factor IX–directed therapy.

**Clinical Experience**

To date, clinical trials investigating IXai have not been conducted. However, factor IXai was used on a compassionate care basis to enable extracorporeal circulation in several patents who could not receive heparin.\(^32\)

**Monoclonal Antibodies**

**Mechanism of Action**

Monoclonal antibodies are currently used for the treatment of cancer, autoimmunity, and thrombosis. Murine antibodies are “humanized” by fusing the variable region or the complementary determining regions of the murine antibody with the heavy chain of human antibodies. Humanized monoclonal antibodies are generally well tolerated.

**Preclinical Data**

Several antibodies against epitopes of factor IX have been developed. A humanized monoclonal antibody directed against factor IX was developed by SmithKline Beecham (presently Glaxo-Smith-Kline). SB 249417 is a chimeric molecule with human IgG1 fused to the complementary determining region of a murine monoclonal antibody BC2, directed against the human factor IX Gla domain.\(^34,35\) In a rat model of arterial thrombosis, the antibody achieved significant reductions in thrombus formation with modest prolongation of the activated partial thromboplastin time (aPTT).\(^35\) In a rat model of stroke,\(^36\) SB 249417 reduced infarct volume and was associated with reduced neurological deficits in animals treated with this antibody compared with animals treated with tissue plasminogen activator. Pharmacokinetic studies in Cynomolgus monkeys showed an elimination half-life of 3.8 days. Suppression of factor IX activity and prolongation of aPTT were rapid and dose dependent.\(^37\)

**Clinical Experience**

A phase I clinical trial with SB 249417 has been completed.\(^38\) The study design was a randomized, single-blind, placebo-controlled, dose-rising trial of single intravenous infusions of the antibody in healthy volunteers to establish the pharmacokinetics and pharmacodynamics of the agent. The antibody displayed a dose-dependent effect on clotting times with a maximal effect at the end of a 50-minute continuous infusion. The safety profile of the compound was not reported.

**RNA Aptamers**

**Mechanism of Action**

Aptamers (L aptus, to fit) are short oligonucleotides (<100 bases) that fold into defined 3D conformations that enable them to bind specifically to a chosen target.\(^39\) Aptamers are identified using an in vitro screening method termed SELEX (Systematic Evolution of Ligands by EXponential enrichment).\(^40\) In general, the SELEX process enables the identification of aptamers with high affinity and specificity for any target protein.\(^41\)

The complex formed between an aptamer and the selected target protein involves a 3D folding of the RNA to yield a surface complementary to the target protein, similar in principle to the manner in which monoclonal antibodies interact with their respective protein targets. Recognition of a target protein by an aptamer can be achieved through multiple types of nucleic acid–protein interactions, including salt bridges, hydrogen bonding, and van der Waals forces.\(^42,43\) Importantly, the 3D fold of the aptamer and the specific interactions used to recognize the target protein are directed by the primary sequence of the aptamer.

Rusconi et al\(^44\) isolated an aptamer (9.3t) specific for factor IXa from a library of 10\(^{14}\) nucleic acid species using SELEX. To prolong the circulating half-life of the aptamer in vivo, polyethylene glycerol (polyethylene glycol-9.3t) or cholesterol (Ch-9.3t) was attached to the 5′ end. Subsequently, they engineered complementary RNA antidotes targeted against various sites of the aptamer. Binding (base pairing) of the antidote to the targeted aptamer changes the 3D fold of the aptamer and thus effectively attenuates the functional properties of the aptamer. Screening the generated antidotes, they identified oligonucleotide 5.2 as the most effective antidote (Figure 3).

**Figure 3.** Structure of the factor IXa aptamer and antidote. The sequence of aptamer 9.3t and its antidote 5.2 are presented (adapted from Rusconi et al\(^45\) by permission from Macmillan Publishers Ltd: Nature, copyright 2002).
Preclinical Data

In vitro binding studies showed that the aptamer binds factor IX, factor X, factor XI, and protein C. In vitro studies showed that polyethylene glycol-9.3t inhibited activation of factor X by factor IXa, and also blocked activation of factor IX by factor VIIa but not factor Xla. Because factor VIIa is thought to bind factor IX via the EGF domains, Gopinath et al suggested that the aptamer may interact with the EGF domains. Studies with human plasma showed polyethylene glycol-9.3t prolonged the aPTT. The antidote oligonucleotide 5.2 was able to reverse 9.3t anticoagulation of human plasma rapidly (<10 minutes) and durably in a dose-dependent fashion.

Animal studies have validated the in vitro findings. Pigs injected with Ch-9.3t developed an elevated aPTT, which was reversed rapidly and durably by administration of antidote 5.2. Aptamer Ch-9.3t prevented thrombosis in a murine model of arterial injury. Supratherapeutic doses of aptamer 9.3t provoked bleeding in a murine tail transection bleeding model, and this bleeding was prevented by administration of antidote 5.2 immediately after tail transection.

In a porcine model of cardiopulmonary bypass, treatment of animals with the factor IXa aptamer/antidote pair was compared with treatment with unfractionated heparin and protamine. The aptamer was associated with an immediate elevation of both plasma and whole blood clotting times but a more modest anticoagulant effect (aPTT 177 s; activated clotting time [ACT] 294 s) compared with unfractionated heparin (aPTT >400 s; ACT >400 s). The clotting times subsequently returned toward pretreatment baseline values within 5 minutes of antidote injection. The study also suggested several advantages of the aptamer/antidote pair over heparin/protamine, including reduced generation of thrombin and inflammatory mediators (interleukin-1B and interleukin-6), and improved cardiac output.

Clinical Experience

A series of multicenter, dose-ranging studies of a modified factor IXa aptamer (RB006) and its complementary antidote (RB007; Regado Biosciences) is currently being conducted. A phase 1a trial randomized 85 healthy volunteers to receive increasing, intravenous boluses of drug (RB006) or placebo followed 3 hours later by an intravenous bolus of antidote (RB007) or placebo. Subjects treated with 15 mg RB006 exhibited a modest increase in aPTT (1.1-fold increase 15 minutes after administration), whereas subjects treated with 30, 60, or 90 mg of RB006 demonstrated a clear response to the drug with mean relative increases in aPTT of 1.3-, 2.1-, and 2.9-fold, respectively. Activated clotting time values followed patterns similar to aPTT values.

Administration of antidote (RB007) resulted in a rapid and durable return of the aPTT value to baseline for all doses of aptamer (RB006), suggesting that administration of RB007 restored factor IX activity levels to within the normal range. On average, neutralization of the pharmacokinetic effect of RB006 by RB007 occurred within 1 to 5 minutes after antidote administration, with no differences between dose levels. Adverse events, specifically bleeding, were similar among placebo, RB006, and RB007 across all dose groups. No major hemorrhagic events were observed.

A phase 1B study has been initiated with patients presenting with stable coronary artery disease and receiving aspirin with or without clopidogrel. A phase 1C study to investigate drug-to-antidote variations and repeated dosing has recently completed enrollment.

Oral Inhibitors

Mechanism of Action

Transotech Pharma developed TTP889, an oral inhibitor of factor IX. Reportedly, the inhibitor is a small-molecule partial inhibitor of factor IXa with little or no activity against factors VIIIa, Xa, Xla, or XIIa. The mechanism of action of this proprietary compound has not been published.

Preclinical Data

The investigators used a rat arteriovenous shunt model to assay the effect of TTP889 effect on fibrin formation. The assay involved analysis of fibrin deposition on a silk thread after rats were exposed to a 15-minute shunt. Rats treated with vehicle deposited 104±43 mg fibrin, whereas rats treated with TTP889 deposited 39±18 mg fibrin (P<0.001). The investigators also compared the efficacy of TTP889 to heparin in a porcine arteriovenous shunt model. In this animal model, pressure across a hemodialysis filter that was shunting blood from the carotid artery to the jugular vein was used as a surrogate for clot formation. Reportedly, 0.3 mg/kg TTP889 functioned as well as 150 U/kg heparin. The group reports that TTP889 did not effect the bleeding time or bleeding volume from cutaneous or splenic incisions in vivo.

Clinical Experience

Transotech Pharma has completed phase I clinical trials at MDS Pharma Sciences in Nebraska. The company reports that the drug was safe at all single and multiple doses and demonstrated a predictable pharmacokinetic profile with a half-life after oral administration of ~20 hours.

The FIXIT Trial is a phase II proof-of-concept clinical trial to determine the safety and antithrombotic efficacy of TTP889 in patients at risk for venous thromboembolism (VTE). This multicenter, placebo-controlled European study has enrolled 300 patients after hip fracture surgery to receive standard prophylactic treatment for 1 week, followed by 3 weeks of extended VTE prophylaxis with daily oral doses of TTP889. To date, results of the FIXIT study have not been published.

Summary and Future Directions

Factor IX inhibitors consistently display effective anticoagulation and reduced risk of bleeding compared with unfractionated heparin (Table). The active-site competitive inhibitor IXai was developed first and evaluated extensively in animal models. However, clinical trials have not followed the encouraging preclinical observations. The monoclonal antibody SB 249417 also established effective anticoagulation in animal models. A phase I clinical trial demonstrated prolongation of coagulation measures in humans, setting the stage...
for subsequent clinical investigation. The oral factor Xa inhibitor TTP889 has undergone early-phase clinical trials and is reportedly well tolerated in humans. Current phase II trials will ascertain safety and provide preliminary effectiveness data in the setting of venous thromboprophylaxis. RNA aptamers represent the newest class of factor Xa inhibitors. The RNA aptamer/antidote pair is an attractive system that produced effective anticoagulation and rapid reversal in animal models and a Phase 1a human study. Clinical trials currently in progress will provide requisite safety, pharmacokinetic and pharmacodynamic information for advanced phase investigation of the drug–antidote pair.

Disclosures

E.L.H. and K.C.D.B. have no conflicts of interest to disclose. R.C.B. is the principal investigator for the factor IXa RNA aptamer and antidote phase Ib clinical trial and receives research support from Regado Biosciences. C.P.R. is a founder and employee of Regado Biosciences (Durham, NC). E.L.H. is currently affiliated with the Department of Pathology, University of Arkansas for Medical Sciences, Little Rock.

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