Factor Xa inhibitors by classical and combinatorial chemistry

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A high-priority goal in the search for new antithrombotic agents is the identification of small-molecule compounds that selectively inhibit the blood coagulation Factor Xa. These compounds should limit blood loss while controlling thrombosis following surgery, heart attacks, infection by Gram-negative organisms, and other coagulation disorders. Three naturally occurring small proteins (antistasin, tick anticoagulant peptide and yagin) and a diverse collection of small-molecule inhibitors of Factor Xa, originating from both structure-based design and screening of historical compounds and combinatorial libraries, have been described. This review summarizes recent entries into preclinical research, including a series of unique Factor Xa inhibitors recently uncovered in combinatorial peptide libraries.

Under normal physiological conditions, blood loss from a damaged vessel will stop within a short period of time (hemostasis). Platelets first adhere to adhesive proteins, such as collagen and von Willebrand factor, in the subendothelial regions of the injured blood vessel; they then aggregate to form the primary hemostatic plug. Platelets stimulate local activation of plasma coagulation factors, leading to generation of a fibrin clot that reinforces the initial platelet aggregate. Later, as wound healing occurs, the platelet aggregate and fibrin clot are degraded. Thrombosis is a pathological form of hemostasis in which a platelet aggregate and/or a fibrin clot occludes a blood vessel. Arterial thrombosis can result in ischemic necrosis of the tissue supplied by the obstructed artery (for example, myocardial infarction resulting from thrombosis of a coronary artery). Venous thrombosis can cause the tissue drained by the vein to become edematous and inflamed. Thrombosis of a deep vein can lead to serious complications such as pulmonary embolism. Thus, identification of therapeutic drugs that maintain hemostasis to limit blood loss while controlling thrombosis following surgery, heart attacks, infection by Gram-negative organisms, and other coagulation disorders is an important goal for the medical community.

Coagulation involves a series of zymogen activation reactions as shown in Figure 1 (Ref. 1). At each stage a precursor protein, or zymogen, is converted into an active protease by cleavage of one or more peptide bonds in the precursor protein. The final protease generated in this coagulation cascade is thrombin (Factor IIa). Thrombin plays multiple roles in coagulation[2]: increasing its own production by efficient conversion of cofactors V and VIII to their active forms (Va and VIIIa); activating platelets by cleaving the thrombin receptor on the platelet membrane; converting fibrinogen to insoluble fibrin; and activating Factor XIII, the transglutaminase that stabilizes fibrin clots by forming γ-glutamyl–ϵ-lysine bridges between fibrin molecules. Thrombin also acts as an anticoagulant, converting protein C to its active form, which in combination with thrombomodulin rapidly inactivates Factors Va and VIIIa.

Because of the central role of thrombin in thrombosis, thrombin inhibitors were an early focus of efforts to develop new and effective antithrombotic regimens[3–5].

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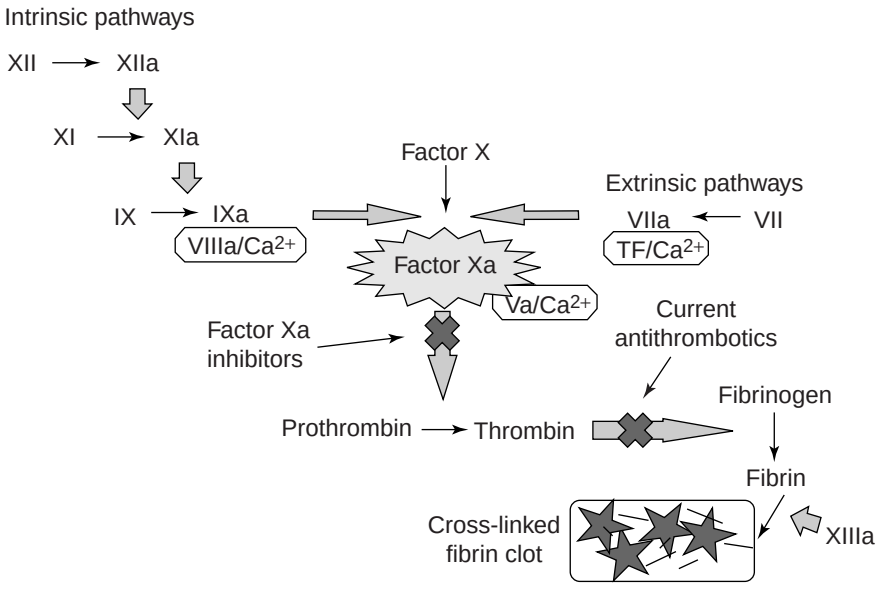

Figure 1. *Blood coagulation cascade and sites for anticoagulation therapy. TF, tissue factor.*

Although heparin has been the drug of choice for many years, it has limited effectiveness in inhibiting clot-bound thrombin, and in some cases produces thrombocytopenia and unacceptable bleeding when administered at antithrombotic doses.

Potent and selective active-site-directed thrombin inhibitors have been investigated as alternatives to heparin, and several are currently in clinical trials. However, direct thrombin inhibitors have shown a tendency to increase the likelihood of bleeding complications, especially when used in combination with thrombolytic agents such as tissue plasminogen activator[5–8]. Another limitation of thrombin inhibitors is that they are unable to block the continuing production of thrombin from prothrombin, which is the most abundant procoagulant zymogen in the circulatory system. The high levels of thrombin inhibition necessary to produce an antithrombotic effect *in vivo* can result in unacceptable levels of anticoagulation and a corresponding risk of hemorrhage. By contrast, inhibition of the prothrombinase complex, which converts prothrombin to thrombin, should prevent the continuing production of thrombin while maintaining a basal level of thrombin activity necessary for primary hemostasis. This concept led to the investigation of ways to inhibit the enzymatic activity of the prothrombinase complex by direct inhibition of its active component, Factor Xa (Refs 9–11).

Factor Xa is a trypsin-like serine protease that converts the prothrombin zymogen to its active form, thrombin (Figure 1). Unlike thrombin, which is known to act on several protein substrates, Factor Xa specifically cleaves prothrombin at two sites, both adjacent to arginine residues. The conservation of Gly-Arg (P_2-P_1) at the cleavage sites was used in the design of various substrates and inhibitors for Factor Xa (Refs 9–12). In addition, unlike thrombin which acts in solution, Factor Xa, together with Factor Va and Ca^{2+} in the prothrombinase complex, normally binds to a negatively charged phospholipid surface (such as on activated platelets or exposed subendothelial tissues). Thus, Factor Xa inhibitors possess theoretical advantages over thrombin inhibitors by acting primarily at sites of injury, preventing the formation of thrombin and by decreasing the feedback loop that amplifies thrombin production.

Currently, a variety of Factor Xa inhibitors are known in the general and patent literature (Figure 2). Several of these have been evaluated in animal models (see below). This review provides a brief overview of current information about active site inhibitors of Factor Xa that are being developed as antithrombotics, and focuses on the recent discovery and optimization of Factor Xa inhibitors in combinatorial libraries. Earlier reviews of Factor Xa inhibitors (prior to 1995) can be found in Refs 9–13.

Naturally occurring Factor Xa inhibitors

The study of the potential role of Factor Xa inhibitors in treating thrombotic diseases was greatly facilitated by the discovery of the medicinal leech-derived inhibitors. Three extremely potent and selective inhibitors of Factor Xa have been described.

Antistasin

A selective and tightly binding inhibitor of Factor Xa called antistasin (ATS) has been isolated from the Mexican leech (*Haementeria officinalis*). Antistasin is a 119 amino acid,

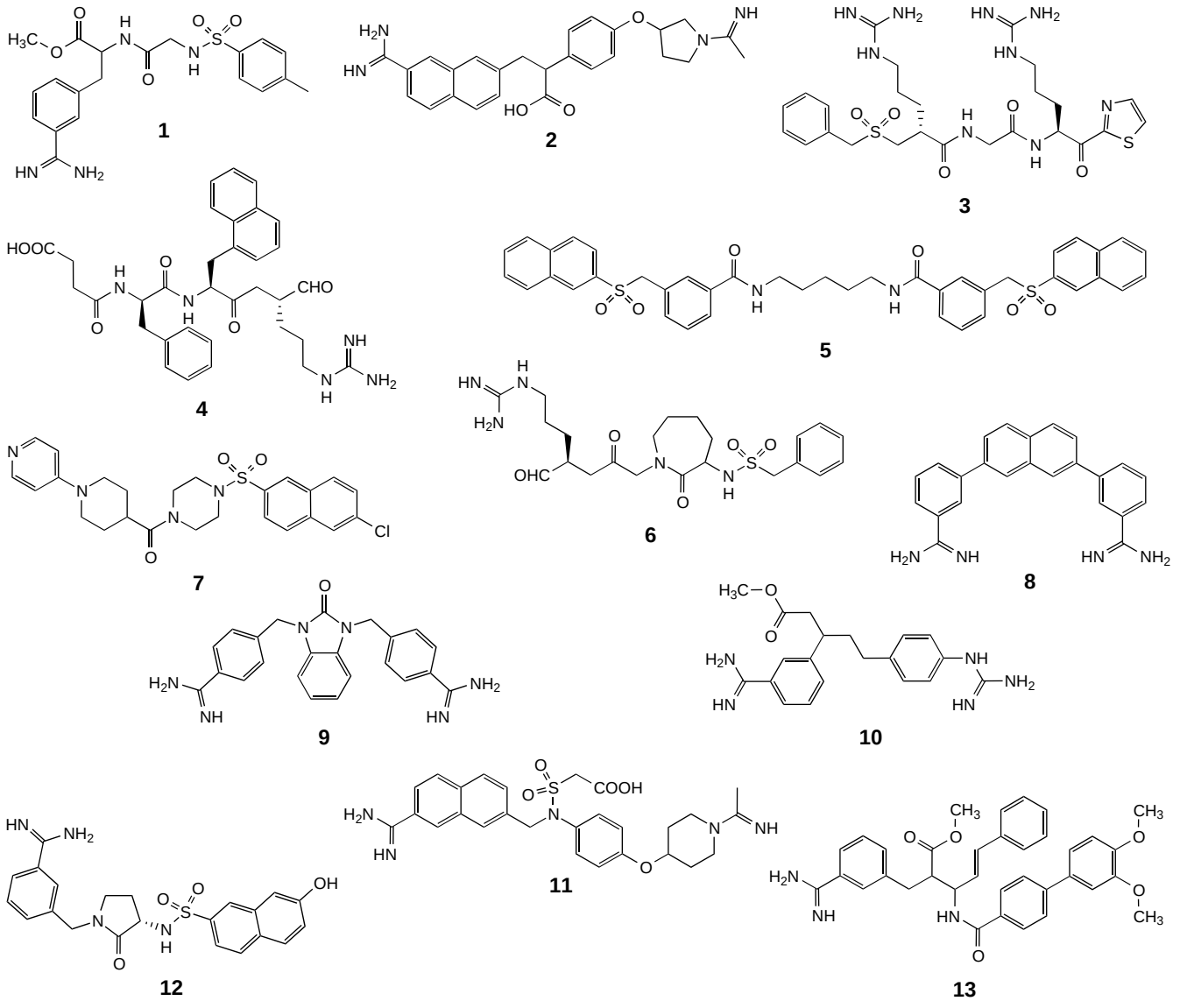

Figure 2. *Known Factor Xa inhibitors.*

cysteine-rich, single-chain polypeptide, the structure and function of which has been reviewed[14]. A recombinant form of antistasin (rATS) has potent antithrombotic activity *in vivo* in a variety of thrombosis models. rATS has a K_i = 43 pM for Factor Xa without measurable effect on thrombin at inhibitor/enzyme ratios as high as 4,000:1 (Ref. 14). Antistasin is cleaved by Factor Xa at a single site, Arg34, forming a slowly dissociating enzyme–inhibitor complex[15]. A series of truncated peptides corresponding to amino acids 27–49 of ATS were evaluated as Factor Xa inhibitors[16]. The most potent peptide synthesized was a disulfide-cyclized, 19 amino acid peptide, $ATS_{29–47}$, which has a K_i = 35 nM against Factor Xa. Removal of the disulfide bridge reduced its inhibitory activity against Factor Xa by more than 95%. The results from this study could assist the future design of small molecule Factor Xa inhibitors.

Tick anticoagulant peptide

A second potent inhibitor of Factor Xa has been isolated from the soft tick (*Ornithodoros moubata*) and designated

tick anticoagulant peptide (TAP). TAP is a 60 amino acid disulfide-linked monomeric protein. Human recombinant TAP (rTAP) has a K_i = 200 pM for Factor Xa inhibition and a 90,000-fold selectivity for Factor Xa over thrombin[14,17]. The structure and function of TAP have been extensively explored[18,19]. In a recent study, site directed mutagenesis showed that replacement of Tyr1 with TrpY1W and Asp10 with ArgD10R increased the inhibitory potency of rTAP against human Factor Xa by more than 2.5- and 4-fold, respectively, while the double mutant, Y1W/D10R, produced an inhibitor with a K_i = 10 pM, a 37-fold enhancement of inhibitory potency toward Factor Xa (Ref. 20). rTAP has been extensively studied in animal models of thrombosis[21–26]. In a direct comparison of rTAP and the thrombin inhibitor hirudin, rTAP was as effective as hirudin in maintaining post-thrombolysis vessel patency in dogs, lending support to the hypothesis that control of ongoing thrombin generation is as effective as direct inhibition of clot-bound thrombin[23]. This study also demonstrated that, unlike hirudin and heparin, antithrombotic efficacy is achieved with rTAP at levels that do not substantially increase activated partial thromboplastin time and template bleeding time.

Yagin

A naturally occurring polypeptide inhibitor of Factor Xa has been isolated from the saliva of the leech *Hirudo medicinalis*[27] and made by recombinant technology. It contains 133 amino acids, including 22 cysteine residues that probably form 11 disulfide bonds. Yagin was tested both *in vivo* and *in vitro* for its effectiveness as an antithrombotic agent. It has been reported that yagin has a half-life of about 80 min in mice, rats and rabbits and of >120 min in baboons[28]. Recently, yagin was compared to heparin and hirudin in a rabbit thrombosis model as an adjunct to thrombolysis with recombinant tissue-type plasminogen activator (rTPA). The results from this study showed that yagin is superior to both antithrombotic agents, with about 50% acceleration of the reflow time relative to that of heparin and hirudin[29].

Small-molecule Factor Xa inhibitors

Tenstop

The design of compound **1** (Tenstop) was based on the conservation of Gly-Arg at the cleavage site of prothrombin by Factor Xa. The synthesis of a family of compounds in which the Arg residue is replaced by Phe substituted on the benzene ring with the basic amidine group has been described[30]. It was shown that the best Factor Xa inhibitors are those with meta-substituted amidinophenylalanine. The retention of Gly at position P_2 with derivatization of its amino group with tosyl sulfonylamide gave compound **1** with a K_i = 840 nM and 16 μM for Factor Xa and thrombin, respectively. This compound was not developed further for clinical testing but is still in use as a research tool.

Daiichi Pharmaceutical DX9065a

DX9065a (**2**) is one of a family of dibasic amidinoarylpropanoic acid derivatives designed as Factor Xa inhibitors[31]. DX9065a is a highly selective Factor Xa inhibitor with a K_i = 41 nM (K_i >2 mM against thrombin), and shows potential as an orally available antithrombotic. The compound showed good inhibition of platelet and fibrin deposition in the formation of venous thrombi but not of arterial-type thrombi[32]. In another study, DX9065a was evaluated for its protective effects in the prevention of thromboplastin- and endotoxin-induced disseminated intravascular coagulation (DIC) in rats. It showed good protection against DIC with no effect on the number of white blood cells or platelets[33]. Recently, X-ray cocrystallization studies of DX9065a with Factor Xa have been reported[34,35]. These studies show that DX9065a binds through the interaction of the naphthylamidine group with Asp189 (S_1 pocket) by a salt bridge and of the pyrrolidine ring with the aryl-binding domain (S_4 pocket) via π–cation interaction[36]. These X-ray results may assist in future design of small-molecule Factor Xa inhibitors.

Cor Therapeutics

Cor Therapeutics reported the synthesis and evaluation of Arg-containing Factor Xa inhibitors[37,38]. The design of compounds **3** and **4** is based on the use of Arg as a P_1 residue with modification of the C-terminus to include an activated heterocyclic group or aldehyde. Also, extension of the N-terminal region to include additional lipophilic or polar residues was used to generate several analogs of the basic structures. Compounds **3** and **4** show inhibitory activity against Factor Xa with IC_{50} = 0.65 nM and 23 nM, respectively[37,38]. The activity against thrombin is 10 μM for **3** and >25 μM for **4**. These compounds have been proposed for the treatment of thrombosis, myocardial infarction and angina.

3D Pharmaceuticals

3D Pharmaceuticals reported that compound **5** has an IC_{50} = 3 μM against Factor Xa with no inhibition of thrombin at 6 μM (Ref. 39).

Corvas International

Recently, a series of transition-state thrombin inhibitors was reported[40]. One of these compounds, CVS1778 (**6**), shows good activity against Factor Xa (IC_{50} = 20.6 nM) but also inhibits thrombin with subnanomolar activity (IC_{50} = 0.71 nM). There are no *in vivo* data for these compounds. Conformational constraint at P_3 enhanced their activity.

Zeneca

A series of structures containing heterocyclic building units[41] has been described. None of these structures contains the guanidino or amidino functionality that was thought to be important for high-affinity interaction with the P_1 site of serine proteases such as Factor Xa. Compound **7** shows an IC_{50} = 3 nM and 34 μM for Factor Xa and thrombin, respectively. Compound **7** and its analogs were evaluated in rat in both *ex vivo* (intravenous dose of 5 mg kg^{-1}) and *in vivo* (arteriovenous shunt model) tests and were reported to show effective inhibition of thrombus formation.

Boehringer Mannheim

A series of bisamidine compounds was synthesized and tested for serine proteinase inhibition[42]. Among the best compounds tested for Factor Xa inhibition was compound **8**. It has a K_i = 1.2 μM and 4.1 μM for Factor Xa and thrombin, respectively. However, it also inhibits trypsin with a K_i = 0.3 μM. Information about the development of these compounds is not available.

Berlex Labs

A series of diarylmethyl cyclic urea containing amidine groups[43] was claimed to show inhibitory activity against Factor Xa. *In vitro* assays for Factor Xa, thrombin and human prothrombinase were reported. Also, *in vivo* activity of these compounds was described. Compounds such as compound **9** are described as active site inhibitors for Factor Xa without specific results.

DuPont Merck Pharmaceutical

A series of bis-phenylamidine compounds was rationally designed as Factor Xa inhibitors[44]. Compound **10** was reported to have a K_i = 9 nM and 3.1 μM for Factor Xa and thrombin, respectively. No further biological evaluation was reported.

Yamanouch Pharmaceutical

A series of 7-amidinonaphthyl-containing compounds, which appear to be based on DX9065a (compound **2**), was described[45]. Compound YM60828 (**11**) shows a K_i = 1.3 nM for Factor Xa with good selectivity (thrombin versus Factor Xa >70,000) towards Factor Xa compared with other enzymes of the blood coagulation cascade. In addition, the hydrochloride salt of **11** shows >20% oral bioavailability in squirrel monkeys.

Rhône-Poulenc Rhorer

A structure-based design series of Factor Xa inhibitors containing a pentacyclic scaffold was described[46]. Compound **12** shows a K_i of 225 nM against Factor Xa and of >4,000 nM against thrombin. A full biological evaluation of this series of compounds is not available.

Rhône-Poulenc Rhorer and Hoffman-LaRoche

A series of non-peptide Factor Xa inhibitors based on a β-amino acid scaffold was described[47]. Compound **13** shows good activity against Factor Xa (K_i = 30 nM) with good selectivity (K_i = 4.7 μM and 2.5 μM for thrombin and trypsin, respectively). No *in vivo* data are available.

Discovery of Factor Xa inhibitors by combinatorial chemistry

From the examples given above, it is clear that protease inhibitor design based on key structural features of natural and synthetic substrates has been successful in producing a wide variety of compounds that inhibit Factor Xa, with varying degrees of specificity for Factor Xa over the closely related proteases thrombin and trypsin. The alternative approach of screening a large array of individual compounds produced in a combinatorial format in the hope of finding new and unexpected motifs that bind tightly and specifically to a protease target has been successful in identifying a new family of Factor Xa inhibitors, and is described below.

Library synthesis

Peptide libraries were designed and synthesized on solid support (Tentagel, Rapp Polymere) using the 'single-bead, single-compound' concept previously described[48]. To generate multiple copies of a single compound on each bead a split–couple–mix methodology was followed[49] (Figure 3).

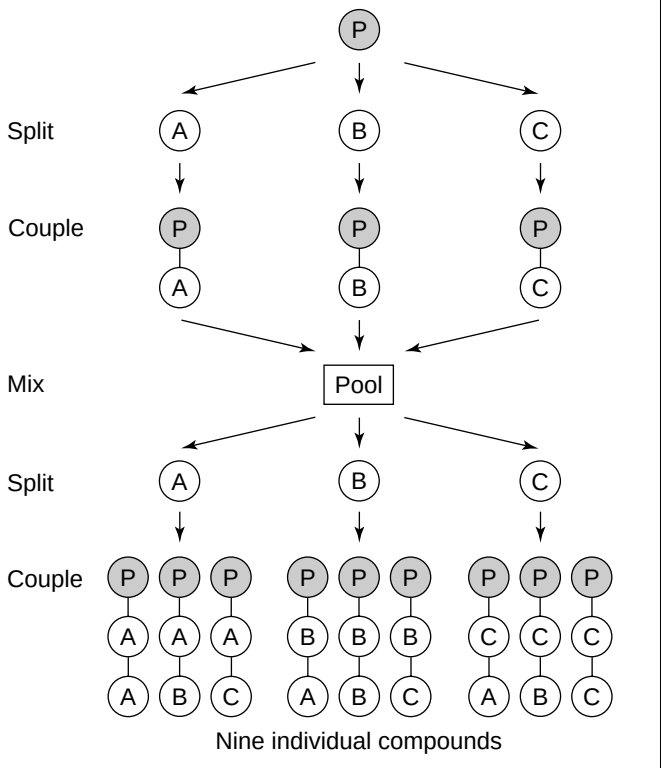

Figure 3. *Split–mix–couple technique of library synthesis. P is a functionalized polymer; A, B and C are building blocks used in library synthesis.*

Library screening

A series of peptide libraries were screened with biotinylated human Factor Xa in a polydentate complex with streptavidin conjugated to alkaline phosphatase (SAP). A solid-phase 'bead-binding' assay (Figure 4), similar in many respects to the widely used Western blot procedure for identifying specific antigens immobilized on nitrocellulose or PVDF membranes, was developed to enable screening of hundreds of thousands of individual peptide ligands per day.

Streptavidin is a tetrameric molecule composed of four identical subunits, each containing a biotin-binding site. By incubating biotinylated Factor Xa in an appropriate ratio with SAP, a tetrameric Factor Xa molecule is produced with greatly increased apparent affinity for binding to peptides exposed on the surface of the Tentagel beads. This can be confirmed empirically by binding assays in which beads containing an inhibitory sequence become rapidly stained when incubated with biotinylated target that has been preincubated with SAP to produce a polydentate probe, while sequential binding of biotinylated target followed by washing and incubation with SAP (to produce primarily monomeric target–SAP complexes) produces weak staining. Beads containing ligands that bind the Factor Xa–SAP complex are identified by incubation (after washing to remove excess unbound probe) in a buffer containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP). This produces a blue precipitate on the exterior of those beads that bind with Factor Xa via active site or exosite interaction with the peptide ligands displayed on the bead surface (Figure 4).

The protocol for the bead-binding assay of Factor Xa was refined using a positive control produced by hydrolyzing the C-terminal methyl ester of Tenstop (**1**, Figure 2; American Diagnostica) and coupling the resulting free acid to amino Tentagel using standard coupling conditions. The resulting positive control beads were used to adjust assay conditions, especially the optimal ratio of biotinylated Factor Xa to SAP during formation of the polydentate complex, to maximize the sensitivity of the assay. Initial screening of peptide libraries returned a large number of sequences with multiple positive charges (arginine, lysine), presumably the result of interaction of the positively charged side-chains with the γ-carboxyglutamic acid (Gla) residues in the light chain of Factor Xa. γ-Carboxyglutamic acid residues coordinate with divalent Ca^{2+} and the negatively charged phospholipid surface of activated platelets to anchor the prothrombinase complex *in situ*, and apparently also have reasonable affinity for positively charged peptides. False-positive controls consisting of linear arginine sequences were used to find conditions that suppressed nonspecific binding to multiple positive charges while retaining binding to the positive controls. Spermidine and Ca^{2+} when included in the incubation buffer suppressed binding to polyarginine control beads without interfering with binding to Tenstop-coupled controls. Consequently, when Ca^{2+} (100 mM) was included in the library incubation buffer binding of Factor Xa–SAP to peptides with multiple positive charges was eliminated.

Identification of ligands that bind only to the active site of Factor Xa was confirmed by a competition step during the screening process. Library beads that stained with Factor Xa–SAP were washed with 6 M guanidinium-HCl to remove bound protein, and destained with dimethylformamide (DMF) to remove color. The beads were then incubated under identical conditions with Factor Xa–SAP that had been preincubated with the reversible inhibitor

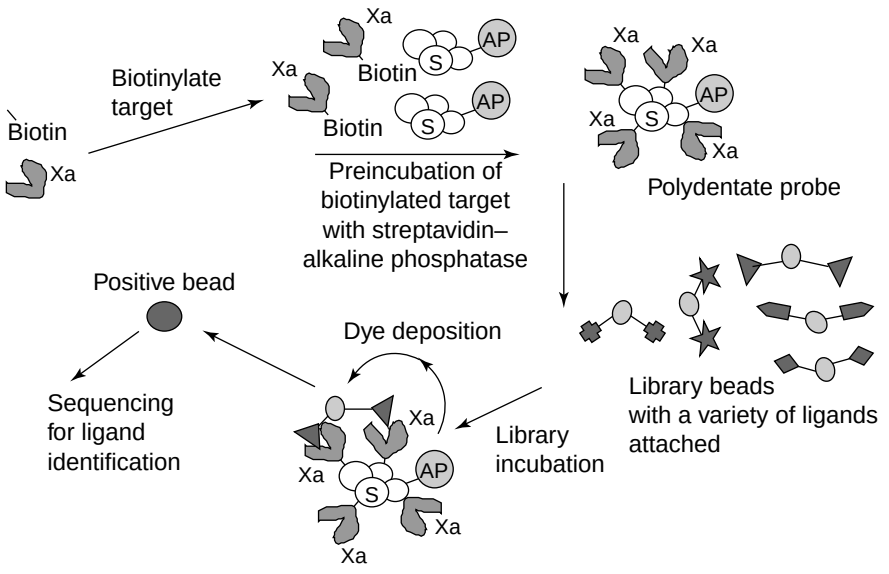

Figure 4. *On-bead screening technique. B, biotin; AP, alkaline phosphatase; S, streptavidin.*

Tenstop, or irreversibly inactivated with the chloromethyl ketone (CMK) PPACK (D-Phe-L-Pro-L-Arg-CMK). Beads that did not stain under these conditions were washed briefly with guanidinium-HCl and DMF and incubated with uninhibited Factor Xa–SAP before color development with the BCIP substrate solution. Beads that restained following this protocol were submitted for determination of the attached peptide sequences by Edman degradation.

Screening of linear D-amino acid, alternating D,L-amino acid, and cyclic D- and L-amino acid peptide libraries was unsuccessful. However, one of the first attempts at screening a linear L-amino acid library produced a series of peptide sequences with an unexpected consensus, which was primarily L-Tyr-Ile-Arg, but in some cases L-Phe-Ile-Arg, at the N-terminus of the peptides[50]. All resynthesized peptides containing the Tyr-Ile-Arg or Phe-Ile-Arg motif inhibited Factor Xa activity in chromogenic assays, with K_i values ranging from 4 to 15 μM, while showing little to no inhibition of the closely related serine proteases thrombin, Factor VIIa/tissue factor, plasmin, tissue plasminogen activator and trypsin. Peptides containing the sequence Tyr-Ile-Arg show competitive inhibition with the chromogenic substrate S2765, yet appear to bind in a nonsubstrate orientation in the active site of Factor Xa (Ref. 50).

A series of deletions of one of the peptides, SEL1691 (Figure 5), showed that removing three to four residues from the C-terminus had little effect on Factor Xa inhibition. Removal of the N-terminal phenylalanine residue or replacement of isoleucine with glycine or alanine eliminated activity of the pentapeptide Tyr-Ile-Arg-Leu-Ala, while replacement of isoleucine with valine or leucine decreased activity more than ten fold. Acetylation increased the activity of the pentapeptide approximately tenfold and, in combination with a twofold enhancement when alanine was replaced by proline, produced a submicromolar (K_i = 485 nM) inhibitor of Factor Xa (SEL2052). Exploration of a limited set of related unnatural amino acids in library format was very informative, showing that a variety of hydrophobic residues, including pyridylalanine, 2-naphthylalanine, *p*-amino-, *p*-chloro- and *p*-fluoro-phenylalanine, and tosylglycine were acceptable replacements for tyrosine. Fewer replacements were tolerated in the isoleucine position, however; one β-branched analog (cyclohexylglycine, Chg) improved activity an additional sixfold to 80 nM in SEL2316 (Figure 5).

Unexpectedly, the combination of a positively charged N-terminal *p*-aminophenylalanine with Chg produced SEL2489 with a K_i = 25 nM and the first indication of prolonged half-life when the compound was administered as an intravenous bolus dose to rats and rabbits. SEL2489 showed anticoagulant activity following intravenous injection in rats and rabbits, with a half-life of 8 and 10 min, respectively. To determine whether loss in anticoagulant activity following injection was the result of adsorption, modification or inactivation by plasma proteins or proteases, SEL2489 was incubated with pooled human platelet-poor plasma or whole blood for 60 min at 37°C before initiating coagulation by the addition of thromboplastin and Ca^{2+}. There was no evidence for loss of activity over time during incubation in plasma or whole blood. Subsequent work with a monoiodinated derivative of SEL2489 showed that elimination in rats occurs primarily via hepatorenal clearance. The half-life was improved further with the replacement of the arginine residue with an

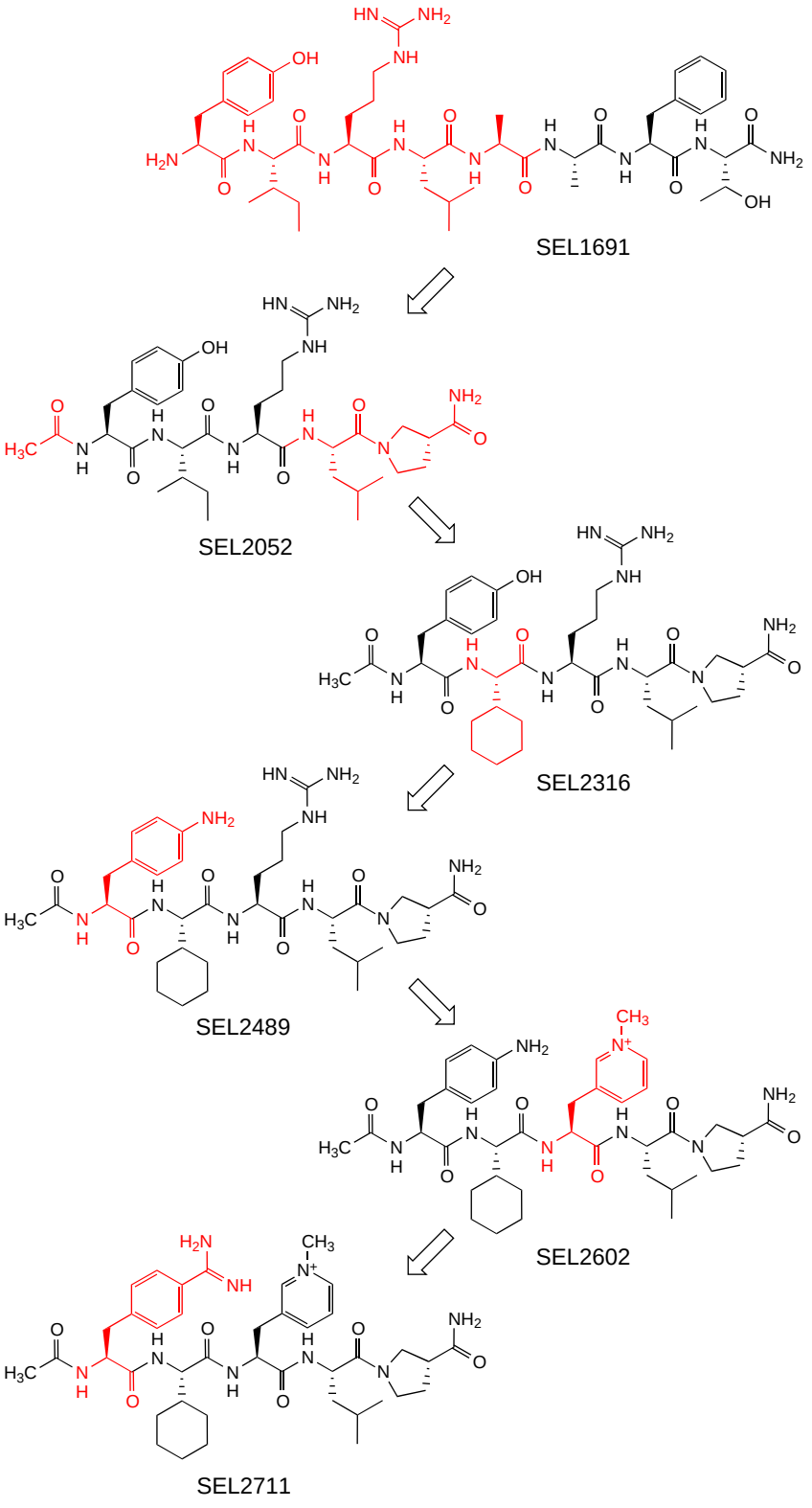

Figure 5. *Optimization of Factor Xa inhibitors.*

arginine mimic, L-3-pyridyl(3′-methyl)-alanine, although the potency of this compound was reduced to a K_i = 285 nM. Activity was restored dramatically when the weakly positive *p*-amino-phenylalanine was replaced by the more basic *p*-amidinophenylalanine (SEL2711). Remarkably, SEL2711 showed both improved potency and half-life when administered to animals, and on further examination was shown to be absorbed and effective in an animal model of thrombosis following intraduodenal dosing.

Conclusion

Current short-term antithrombotic therapy consists primarily of the administration of two relatively nonspecific drugs: heparin or low molecular weight heparin to inhibit the production of fibrin-rich thrombi; and aspirin to minimize platelet aggregation. Newer, more specific, antagonists of platelet aggregation (i.e. RGD derivatives) and fibrin formation (active-site-directed thrombin and Factor Xa inhibitors) are needed to provide safer and more effective anticoagulant treatment with minimal side effects.

Traditional biochemical and medicinal chemistry approaches have been effective in developing a variety of potent and specific Factor Xa inhibitors; however, at present there are only limited descriptions of the discovery and development of Factor Xa inhibitors by screening combinatorial libraries. Clearly, the strength of the approach lies in identifying new and unexpected small-molecule ligands by the 'brute force' method in which the target is presented with a diverse and unbiased collection of structures. Although this at first appears to be the antithesis of another major effort in the pharmaceuti-

cal industry – rational drug design – the two approaches are complementary and are, in fact, natural partners. The combination of high throughput screening of combinatorial libraries, to identify new and proprietary ligands for cocrystallization with disease targets, with further modification by synthetic medicinal chemistry, promises to extend the usefulness of both approaches to drug discovery well into the future.

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