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Review Article

Progress in the design of inhibitors of coagulation factor Xa

William R. Ewing*, Henry W. Pauls and Alfred P. Spada

Rhône-Poulenc Rorer, Department of Medicinal Chemistry, 500 Arcola Road, Collegeville, PA 19426, USA.
**Correspondence*

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Introduction

Myocardial infarction, stroke, deep vein thrombosis and pulmonary embolism accounted for approximately 2 million deaths in the United States in 1996 (1). The formation of an occlusive thrombus is causally related to the pathology of these conditions. As such, antithrombotic therapy is a crucial component in both acute intervention procedures and chronic prevention strategies for treatment and management of these diseases.

Effective antithrombotic therapy often requires administering a combination of antiplatelet and anticoagulant agents. Aspirin and heparin represent the current mainstay of combination therapy for the treatment of coronary syndromes (2). The shortcomings of each have driven intense efforts within the pharmaceutical industry to identify and develop more effective and safer antiplatelet and anticoagulant agents. Significant advances have been made in the development of more potent and effective antiplatelet agents (3, 4); however, the clinical need for improved anticoagulant agents is arguably greater. Current treatment options are limited to unfractionated heparin (UFH), low molecular weight heparins (LMWHs) and warfarin. The challenge remains to achieve consistent, predictable and clinically effective levels of anticoagulation while minimizing the risk of bleeding complications.

Recent approaches to identify anticoagulant agents with improved safety and efficacy have focused on developing specific inhibitors of enzymes within the coagulation cascade (5). Thrombin remains the most extensively investigated of these targets. Two decades of experience in the design of thrombin inhibitors has led to the development of highly potent and selective inhibitors, several of which have been investigated in large-scale clinical studies (6-10). In general, the results of these studies have fallen short of expectations, demonstrating no clear advantage over heparin in the treatment of coronary syndromes (11). More encouraging clinical results have been obtained in the treatment of venous thromboembolism (12).

Factor Xa inhibitors, the subject of this review, represent a more recent and rapidly evolving approach toward the development of anticoagulant agents. Theoretically, direct inhibition of factor Xa activity should provide a potent anticoagulant devoid of the potentially limiting side effects observed with thrombin inhibitors (5, 13).

Background

To develop superior anticoagulant strategies requires an understanding of the biochemical and biophysical mechanisms activating and regulating blood coagulation. The process of normal hemostasis requires maintaining a delicate balance between the dynamic processes of pro- and anticoagulant activities in circulating blood. Both are governed by a finely tuned and highly integrated cascade of enzymatic processes that amplify the response to vascular injury preventing hemorrhage and initiating the repair processes. An abbreviated scheme highlighting the key aspects of these processes is presented in Figure 1.

Two convergent procoagulant pathways have evolved, each capable of being activated in response to different stimuli. The intrinsic and extrinsic coagulation pathways consist of a cascade composed of serine proteases which effectively amplify the initial stimulus to provide a strong and rapid signal to initiate the coagulation process (14). These pathways ultimately converge upon the formation of factor X and its conversion to factor Xa in the prothrombinase complex.

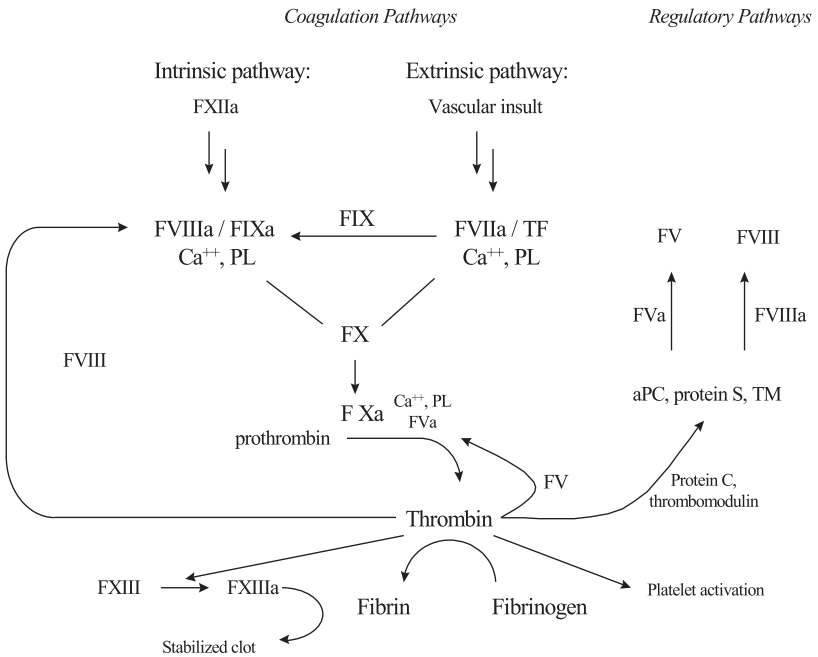


Fig. 1. Schematic diagram of the enzymatic processes of procoagulant and anticoagulant activities in circulating blood. FXa = factor Xa, PL = phospholipid, TF = tissue factor, TM = thrombomodulin, aPC = activated protein C.

The intrinsic pathway is stimulated by the contact of flowing blood with foreign surfaces or upon vascular injury and exposure of subendothelial matrix collagen (15). Three circulating factors, high molecular weight kininogen, prekallikrein and factor XII, bind together on the surface to yield the catalytically active serine protease, factor XIIa. This branch of the coagulation cascade ultimately produces factor IXa. Factor IXa combines with factor VIIIa, phospholipid, Ca^{2+} and factor X in the intrinsic Xase complex to afford factor Xa.

The extrinsic pathway is initiated following vascular injury and the resulting exposure of tissue factor (TF) onto the surface of endothelial cells and macrophages (16, 17). Tissue factor binds to factor VII and catalyzes its conversion to factor VIIa. This complex, in the presence of Ca^{2+} and phospholipid, converts factor X to factor Xa. Factors XI and IX can also be activated by tissue factor/factor VIIa, thus providing a link with the intrinsic pathway.

Factor Xa, generated by either pathway, combines with the nonenzymatic cofactor Va and Ca^{2+} on the phospholipid surface of platelets or endothelial cells to form the prothrombinase complex. The catalytic activity of factor Xa in this complex is increased 300,000-fold relative to its activity in solution. Factor Xa in the prothrombinase complex converts prothrombin (factor II) by limited proteolysis to release catalytically active thrombin (factor IIa) (18).

Thrombin holds a central position in control of coagulation processes. It catalyzes the cleavage of fibrinogen to fibrin, thus initiating the process of clot formation and activates factor XIII which cross-links fibrin monomers to stabilize the developing clot. Thrombin binds avidly to fibrin and remains catalytically active within the growing thrombus (19). Thrombin also catalyzes the formation of cofactors Va and VIIIa and thus provides a positive feedback mechanism for its continued generation and the progression of clot development (20). In addition to its direct actions on the coagulation cascade, thrombin is also a potent agonist of platelet activation. The direct activation of platelets is an important first response to maintain normal hemostasis, allowing the generation of a homeostatic plug following injury (20). Prolonged activation and recruitment of platelets will further potentiate the development of clot formation.

Thrombin also plays a key role in the initiation of inhibitory pathways to downregulate the coagulation process. Upon release from the prothrombinase complex, thrombin can bind to thrombomodulin, a glycoprotein present on the surface of endothelial cells (22, 23). Thrombin has high affinity for thrombomodulin which alters its substrate specificity from fibrinogen and the procoagulant factors V, VIII and XIII to protein C. Cleavage of protein C bound to thromomodulin affords activated protein C. This complex, in the presence of the nonenzymatic cofactor protein S, cleaves the procoagulant cofactors Va and VIIIa, thus providing a mechanism of inactivating the intrinsic coagulation pathway. In addition, active protein C activates the fibrinolytic system by stimulating the release of tissue plasminogen activator from endothelial cell, which provides a physiologically important link between anticoagulant and fibrinolytic pathways (24).

Tissue factor inhibitor controls the regulation of the extrinsic pathway. This protein is an active-site inhibitor of factor Xa that binds with tissue factor/factor VIIa to form a quaternary inhibitor complex (25, 26). Tissue factor inhibitor is synthesized by endothelial cells of which approximately 80% remains associated with the endothelial surface (25).

Antithrombin III (ATIII) is an important circulating endogenous inhibitor of coagulation that acts in addition to the two dynamic regulatory mechanisms discussed above. ATIII inhibits plasma thrombin, factor Xa and, to a lesser extent, IXa activities. ATIII binds irreversibly to these serine proteases to form 1:1 inhibitory complexes. The rate of this inhibition, which is relatively slow otherwise, is accelerated 4000-fold upon binding with the glycosaminoglycan, heparin (27). Heparin, which is synthesized in the liver, is an integral cell surface component which is exposed to flowing blood on the surface of endothelial cells. Therefore, under conditions of normal hemostasis, ATIII is bound and activated by heparin on the endothelial cell surface as well as within the subendothelium, sequestering and inactivating circulating procoagulant enzymes thrombin and factor Xa (28).

In clinical practice, the use of unfractionated heparin (UFH) has been a part of standard anticoagulant therapy for several decades (29). The ATIII-heparin complex effectively neutralizes soluble thrombin but, owing to the size of the formed complex, it is incapable of inhibiting thrombin activity once bound to fibrin in the growing thrombus (19). This physiochemical limitation significantly restricts the ability of heparin to maximally inhibit the actions of thrombin. It is estimated that approximately 40% of thrombin generated within the developing thrombus remains bound to the clot and is therefore resistant to inhibition by heparin-ATIII (30). In addition, thrombin bound to soluble fibrin fragments produced during thrombolysis is also protected from inhibition but still remains capable of activating platelets and coagulation (31, 32).

Heparin therapy is associated with a number of well-recognized risks and disadvantages, several of which are highlighted below (29). Firstly, the anticoagulant response to heparin varies widely among patients. As a result, treatment with heparin requires close monitoring to maintain effective and safe levels of anticoagulant activity. The lack of predictability and the need for continuous monitoring prevents the use of UFH outside of the hospital setting. Secondly, it is estimated that approximately 3% of patients treated with UFH will develop a severe and potentially life-threatening form of heparin-induced thrombocytopenia (33). Lastly, a prothrombotic “rebound” phenomenon has been observed following the termination of heparin treatment, which results in recurrent episodes of unstable angina (34). In a separate study, it was demonstrated that this reactivation phenomenon is the result of increased thrombin activity following termination of heparin infusion (35). The precise mechanism for the rebound phenomenon is not clear but it has been suggested that continued thrombin generation, along with other procoagulant factors, during the course of treatment may play a role (35).

Low molecular weight heparins (LMWHs) offer distinct advantages when compared with UFH (36, 37). They provide predictable and well-controlled anticoagulant response with fixed-dose administration. The bioavailability and half-life of LMWHs following s.c. administration are generally good, allowing for once- or twice-daily dosing (38). Combined, these attributes enable LMWHs to be used safely outside of the hospital setting and avoid the need for continuous patient monitoring. Additionally, one LMWH, enoxaparin, has demonstrated superior efficacy when compared with heparin in several clinical trials (39, 40). It is interesting to note that not all LMWHs afford the same level of efficacy when compared with standard heparin (37, 41). This may be the result of different anti-Xa/anti-IIa ratios, ranging from 1.9:1 to 5.0:1, depending upon the specific product preparation (37, 41, 42). As with heparin, however, LMWHs do not inhibit clot-bound thrombin or platelet bound Xa nor are oral formulations available (36).

Warfarin is the only orally active anticoagulant currently available. It inhibits the vitamin K-dependent conversion of glutamic acid to gamma-carboxyglutamic acid residues of the procoagulant factors II, VII, IX and X and anticoagulant proteins C and S. This posttranslational modification, which occurs in the liver, is essential for the Ca^{2+} and membrane binding properties of these proteins without which catalytically effective complexes cannot be formed. The onset of action of warfarin is slow and often accompanied by a paradoxical increase in coagulation activity (43). This reflects the shorter half-lives of the anticoagulant proteins C (6 h) and S (30 h) as compared with the half-lives of factors II (60 h), VII (5 h), IX (24 h) and X (40 h) (44). As a direct consequence of this mechanism of action, it may take 72-96 h to achieve peak anticoagulant activity. Warfarin possesses a high oral bioavailability (~ 100%) with a long terminal half-life (~ 1 week). As a result, the anticoagulant activity of warfarin may continue for several days following the termination of treatment. Warfarin has a narrow therapeutic margin with many known drug-drug interactions which requires that patients be treated individually and continually monitored for coagulant activity (45).

Numerous preclinical and clinical studies have demonstrated that direct low molecular weight thrombin inhibitors are capable of effectively blocking clot-bound thrombin activity (46). Therefore, superior efficacy should be expected from direct acting inhibitors relative to heparins (11). The results of clinical trials with these agents, however, have generally been disappointing and portend several potential limitations associated with this approach (11, 47). Two large-scale studies conducted with hirudin, GUSTO-IIa and TIMI-9A, were terminated due to excessive cranial bleeding (48, 49). Both studies were reinitiated (GUSTO-IIb and TIMI-9B) with lower doses of hirudin and heparin (50, 51). Unfortunately, hirudin did not demonstrate a statistical improvement over standard heparin therapy in either of these studies at their respective 30-day combined primary endpoints. Clinical data obtained with inogatran and argatroban

Table I: Potential advantages of factor Xa inhibitors.

Inhibition of the source of thrombin generation rather than its catalytic activity
No direct effect upon thrombin-activated platelet aggregation, thus minimizing bleeding risk
No direct effect on thrombin-mediated generation of aPC-via fIIa/TM complex
Minimum risk of thrombotic reactivation rebound and associated ischemic events

have been similarly disappointing (52-54). In addition, cessation of treatment with either agent was associated with thrombotic rebound which led to increased coronary events (52, 55). At the present time, hirudin is approved in Europe for the prevention of deep vein thrombosis following hip and knee replacement surgery (56). Argatroban is approved in Japan for peripheral vascular disease and acute cerebral infarction (54).

The design of direct-acting thrombin inhibitors has been the subject of several recent reviews (8, 10). Many highly potent and selective inhibitors have been described. However, until recently, combining these essential features into inhibitors with strong oral pharmacokinetic properties has remained elusive (57). Sanderson *et al.* recently reported a series of potent thrombin inhibitors with very encouraging pharmacokinetic properties (58). This may herald an important advance in the development of safe and effective oral anticoagulants to replace warfarin. Although there are many earlier reports of orally active thrombin inhibitors, the actual pharmacokinetic profiles of these agents are rather weak. This may be particularly important since clinical data suggests that long-term and/or prophylactic anticoagulant therapy can provide a significant benefit over current standard treatment (59).

Direct inhibition of factor Xa activity in the prothrombinase complex blocks the single physiological source of thrombin generation. Inhibiting the source of thrombin generation rather than its catalytic activity offers several potential mechanistic advantages that could afford superior anticoagulant agents. Direct inhibition of factor Xa activity should have minimal impact upon normal hemostatic response/regulation processes. Platelets, for example, would remain responsive to the low levels of catalytically active thrombin and thus, the formation of platelet hemostatic plugs would not be compromised. As a consequence, the risk of bleeding complications might be minimized. The endogenous pathway to downregulate thrombin production via the thrombin/thrombomodulin complex would also remain intact. The risk of provoking prothrombotic rebound episodes observed with heparin and thrombin inhibitors would be minimized as well (Table I).

The prospects for factor Xa inhibitors in therapy, especially in contrast to thrombin inhibitors, were examined previously in this journal (5). The most recent comprehensive review of factor Xa inhibitors focused on the potential of a combinatorial approach to identifying factor Xa inhibitors (60). Factor Xa inhibitors have also been included in the Annual Reports of Medicinal Chemistry since 1995 (61). Notwithstanding the potential advantages, the field of factor Xa inhibition has not reached the same level of maturity as has thrombin inhibition. Although academic interest in the inhibition of factor Xa has a long history, drug companies had not taken up the challenge in force until the early part of this decade. Given their similarities, some of the design features used in the development of thrombin inhibitors were adapted and incorporated into the design of factor Xa inhibitors. However, in contrast to thrombin, small molecule inhibitor/factor Xa X-ray structures have been published on only two occasions, the first one appearing in 1996 (73). Thus, structure information has only begun to have an impact on the design of factor Xa inhibitors as compared to thrombin inhibitors (8). Nonetheless, much progress has been made in recent years and will be discussed from an historical perspective using representative examples.

Factor Xa structure and function

Human factor Xa consists of two chains (62): a heavy chain which incorporates the catalytic triad (63) and a light chain which contains a chymotrypsin cleavage site, Tyr44-Lys45 (64). Structural information for factor Xa was first obtained by Tulinsky *et al.* (65) on a large molecular fragment of the enzyme generated by autocatalysis during crystallization studies. The des(N-terminal 1-45) factor Xa X-ray structure was solved to 2.2 Å resolution and revealed that the side chain of the C-terminus Arg439 was inserted into the S1 specificity site of a neighboring molecule of factor Xa. This ruled out small molecule inhibitor work using classical soaking techniques. It is also worth noting that this form of the enzyme lacks associated factor Va, which is necessary for large rate enhancements observed *in vivo* (18). However, the structure was useful for modeling studies and in fact several groups took advantage of this approach for inhibitor design (see below).

The catalytic triad residues, Ser195, His57 and Asp102, provide the catalytic machinery for the hydrolysis of the peptide linkage of the substrate. The mechanism of this process has been extensively studied for serine proteinases in general (66, 67). Initial binding between enzyme and substrate is followed by nucleophilic attack on the amide carbonyl to form a tetrahedral intermediate (Scheme 1A). Collapse of this intermediate gives the acyl enzyme and liberates the amine and subsequent hydrolysis yields the acid. Interaction with the nucleophilic components of the triad, *i.e.*, the serine hydroxyl and the histidine imidazole, has been a general approach for the inhibition of serine proteinases (68).

The β-sheet, which stretches across the middle of the Xa active site, is also found in thrombin and trypsin. Proteinaceous and peptide-based substrates and inhibitors of factor Xa often bind in an extended conformation. In this standard binding mode the peptide backbone engages in H-bonding interactions with the Gly216 residue of the β-sheet (69). Tick anticoagulant protein (TAP) appears to be an exception (70).

The specificity or S1 binding pocket of the trypsin-like serine proteinases is one of the prime determinants of substrate specificity. In factor Xa, the S1 pocket is a narrow cleft with an aspartate residue at its base. Except for small changes, this feature is also found in related serine proteinases such as trypsin (71) and thrombin (72) and, as such, presents a challenge for the design of specific inhibitors. The aromatic or S4 binding pocket of factor Xa is one of its unique structural features. The floor and walls of this open-ended box are defined by two electron-rich aromatic side chains (Trp215, Tyr99) and Phe174. The terminus of the box is lined with H-bond acceptors, *i.e.*, the carbonyl functions of Lys96 and Glu97 which bind a structural water and an acidic side chain, located at the periphery (Glu97). This unique juxtaposition of functionality, termed the "cation hole", has been invoked to explain the potency of inhibitors with positively charged moieties at the putative P4 or P3 position (73).

Given the apparent difficulty of obtaining small molecule/factor Xa crystal structures, various groups have resorted to using the related serine proteinases trypsin (74, 75) and thrombin (76) as surrogates for factor Xa. This is possible since the factor Xa inhibitors of interest often retain some level of activity against these enzymes. Trypsin has been used more frequently in this effort due to its availability and higher degree of homology to factor Xa (77). Even so, differences exist in both the S1 and the S4 pockets. The most significant difference in S1 is an Ala190 (factor Xa) to Ser190 (trypsin) variation. This suggests that in factor Xa the S1 subsite is somewhat more hydrophobic than the trypsin S1 pocket. The differences in S4 are the most profound involving Phe174 and Tyr99 (factor Xa) to Glu174 and Ile99 (trypsin) mutations, respectively. Although the possibility for strong π-cation interactions (78) are greatly diminished in trypsin *versus* factor Xa, cations are accommodated in the S4 pocket of trypsin (74, 75).

Affinity labels

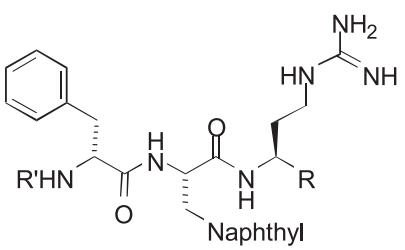
Affinity labels or active site-directed inhibitors such as chloromethyl ketones have been used for several decades to irreversibly inhibit serine proteinases (79). The factor Xa inhibitor dansyl-Glu-Gly-Arg-chloromethyl ketone is a typical example (80). The presumed mechanism of inhibition involves formation of a hemiketal between Ser195 and the electrophilic carbonyl; subsequent nucleophilic attack by His57 on the chloromethyl yields covalently and irreversibly bound inhibitor (Scheme 1B). Tulinsky has modeled this interaction showing that the dansyl group fills the S4 pocket and the specificity pocket is occupied by the arginine side chain (65). Given the inherent reactivity of affinity labels in general, this approach is out of favor as a viable strategy for drug design.

Transition state analogs

Another strategy for the inhibition of serine proteinases has been to replace the scissile bond of a truncated peptidyl substrate with electrophilic functions such as boronic acids, aldehydes or activated ketones (81). The covalent adduct which results upon incubation with enzyme, although reversible, is tightly bound because it mimics the transition state formed during catalysis (Scheme 1C). Proteinaceous substrates for factor Xa bound in the prothrombinase complex include prothrombin (82) and factor V and several inhibitors such as antistasin (83), AcAP-6 (84) and tissue factor pathway inhibitor (85). The active site sequences of these ligands have been summarized (86). Except for AcAP-6, all incorporate a P1 arginine and are thought to inhibit factor Xa in the extended substrate conformation. AcAP-6 has a phenylalanine P1, suggesting a tolerance for aromatic rings in the S1 subsite. The P4 residue is usually hydrophobic except for factor V which contains a lysine.

A number of laboratories have taken this approach for the design of factor Xa inhibitors and representative examples are given below. Several of the first transition state analog inhibitors of factor Xa described incorporated arginine at P1 and aromatic residues at P2 and P3. Aldehydes **1** (87) and boronic acids **2** (86) are effective inhibitors of factor Xa. A series of peptidyl inhibitors have been described which incorporate arginines at P1 and P3 and which utilize ketoamides **3** (88) or ketothiazoles **4** (89) as serine traps. In general, the presence of the serine trap has a profound impact on the activity of these peptide derivatives and underscores the potential for potency enhancements in trypsin-like serine proteinases via catalytic triad interactions (90).

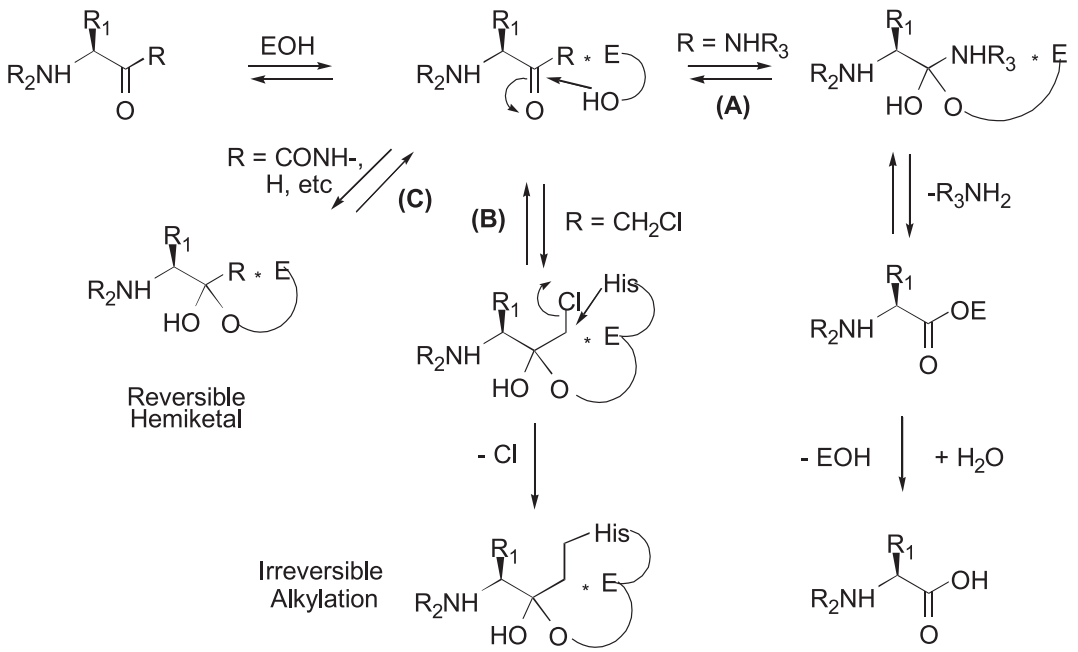
Assuming that interaction of the electrophilic function with the catalytic triad anchors the inhibitor and that the P1 arginine is inserted in the S1 subsite, it is noteworthy that both basic and aromatic groups are tolerated at the

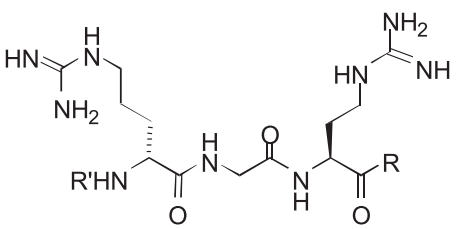


(1) R=CHO, R'=$C(O)CH_2CH_2CO_2H$

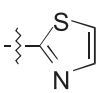
(2) R=$B(OH)_2$ R'=Ac

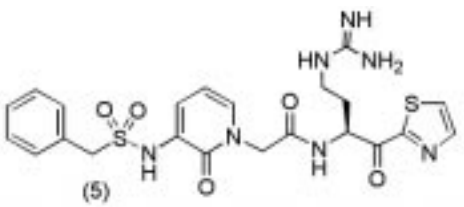
Scheme 1: Serine Proteinase Hydrolysis and Inhibition



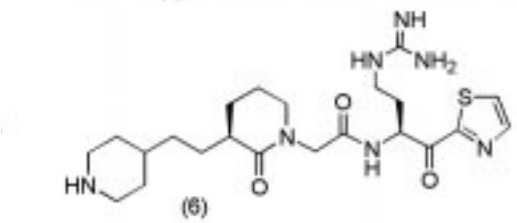


(3) R=C(O)NHBn, R'=SO_2Bn

(4) R=  R'=SO_2Bn



(5)



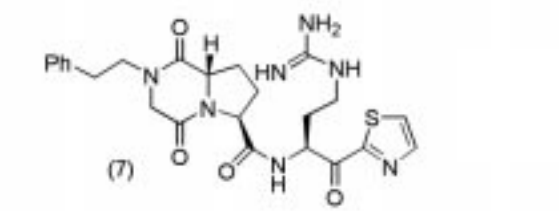
(6)

putative P3/P4 position(s). It has been suggested that basic groups such as arginine could take advantage of a π-cation interaction with the S4 subsite (78).

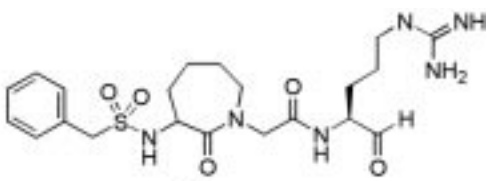
Several companies have taken this concept a step further by incorporating a dipeptide mimetic as a scaffold for delivery of P1, the serine trap and the P4 ligand. This strategy has been employed with good success in the area of thrombin inhibition (8). One might assume that critical H-bonding interactions are maintained for these inhibitors as is seen in similar thrombin inhibitor structures. Representative examples from the patent literature which claim anti-Xa activity include pyridones **5** (91) and δ-lactams **6** (92), both from Cor Therapeutics, Warner Lambert's pyrrolopyrazinediones **7** (93) and ε-lactams **8** (94) from Corvas. The Corvas compound **8** is representative of a large number of published thrombin inhibitors (94) and is more potent against factor IIa (Ki = 0.71 nM) than factor Xa (Ki = 21 nM).

Peptide inhibitors

A number of polypeptide inhibitors of factor Xa, whether natural, mutations or synthetic, have been described (95). For the purposes of this review, we will restrict our comments to selected smaller members of this class being pursued as therapeutic agents. Using a combinatorial approach, the Selectide group described a series of pentapeptides (*e.g.*, SEL-2052, (**9**) Ki = 485 nM) derived from Tyr-Ile-Arg (YIR)-containing octapeptides (96). That the YIR structural motif was found in this manner is remarkable, especially in comparison with the recently published X-ray structure of TAP complexed to



(7)



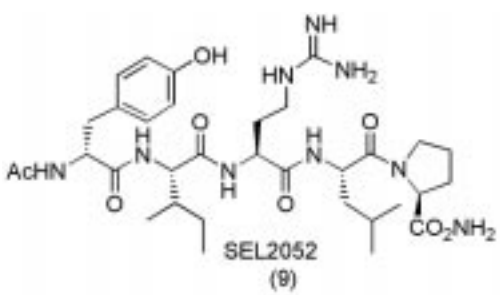
(8)

bovine factor Xa. A tyrosine residue is bound in the S1 pocket and an arginine is located in the S4 pocket (70).

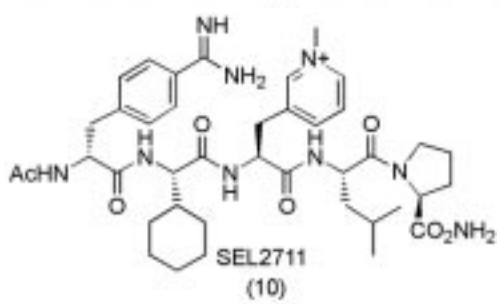
Further optimization by the incorporation of nonnatural amino acids led to SEL-2711 (**10**) (Ki = 3 nM) (60). Assuming a substrate-like binding mode, the unique feature of this inhibitor is the putative P1 residue, a methyl pyridinium salt. Alternatively, a binding model of SEL-2711 in factor Xa has been proposed wherein the benzamidine function is placed in S1 (97). The amidine forms a salt bridge with Asp189 while the methylpyridinium group is bound into the S4 subsite, a potential π-cation interaction. No binding interactions have been ascribed to the large hydrophobic P2 residue. Interestingly, SEL-2711 demonstrated improved potency and half-life as compared to earlier members of this series and was found to be effective in animal models of thrombosis when administered interduodenally (60).

Bis-benzamidine inhibitors

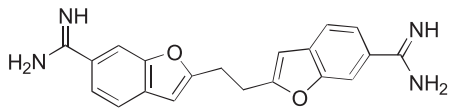
The identification of nonpeptide factor Xa inhibitors has been the continuing goal of many pharmaceutical



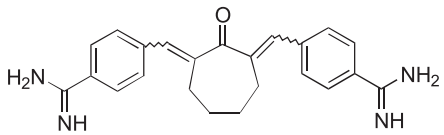
SEL2052
(9)



SEL2711
(10)



DABE (11)

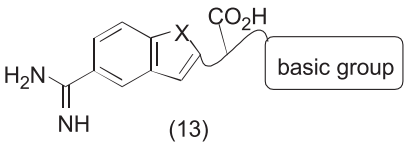


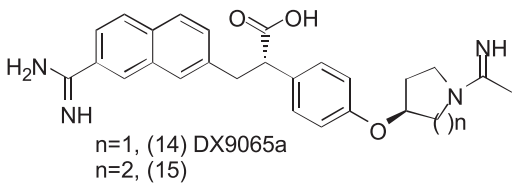
BABCH (12)

research efforts. Most of the recently published approaches have included modification of previously known inhibitors, optimization of screening leads or rational drug design using the crystal structure of des(1-45)-factor Xa (65). The starting point for several recently described approaches began with modifying two bis-benzamidine inhibitors of factor Xa, DABE (**11**) (98) and BABCH (**12**) (99). The reported Kis of DABE and BABCH for bovine factor Xa are 570 nM and 13 nM, respectively.

One of the first highly potent, selective and orally active nonpeptide inhibitors of factor Xa is DX-9065a, reported by the Daiichi group (100-102), with DABE used as the starting point for their drug discovery process. The design strategy assumed that for the symmetrical inhibitor, one benzamidme group inserted into S1 while the other resided in S4. Also noted was the improved oral absorption profile of zwitterionic benzamidines such as 4-amidinophenylpyruvic acid (APPA) (100, 103). By combining these structural features, they envisioned a bicyclic amidine tethered to a basic group by a spacer containing a carboxylic acid. Optimization studies on this general construct **13** led to the identification of DX-9065a **14** as a potent inhibitor of factor Xa (Ki = 41 nM) with high selectivity over thrombin (Ki > 2000 nM). The X-ray crystal structure of DX-9065a bound to the active site of factor Xa was solved (73), revealing several interesting structural features. As anticipated by models, the naphthamidine group was bound in the S1 pocket. The amidine, however, forms only one H-bond with Asp189. The other nitrogen of the amidine is hydrogen bonded to the carbonyl oxygen of Gly291. The pyrrolidine ring is inserted deep within the S4 pocket with the acetimidoyl group in the cation hole at the back of the pocket. The carboxylic acid of the inhibitor was found to form a weak H-bond with Gln192. DX-9065a does not form an H-bond with Gly216, an interaction found for peptidomimetic inhibitors of serine proteinases such as thrombin and trypsin (69).

The Yamanouchi group further improved upon the potency of DX-9065a (104). Their starting point was the Daiichi piperidine analog **15** that was found to be 3-fold more potent *in vitro*. The benzylic carbon was replaced by a nitrogen resulting in an achiral series. The simple case where R=H was found to be 2-fold more potent than DX-9065a. The series was expanded by linking the R group as an amide, urea, urethane, sulfonamide and sulfonyl

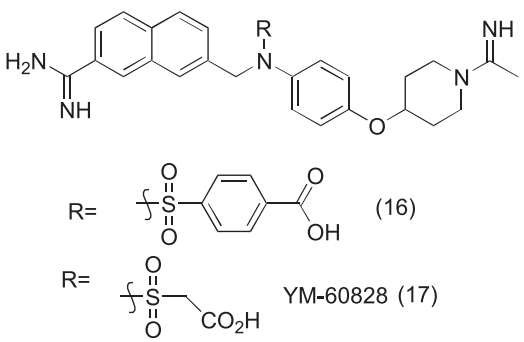


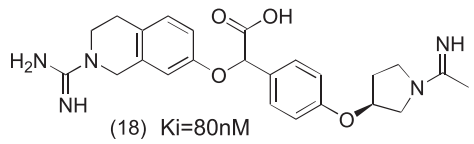


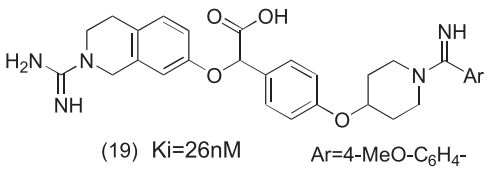
urea. Sulfonamide linked R groups gave the most promising results, with compound **16** being the most potent (IC_{50} = 0.07 nM). Profiling compounds from this series *in vivo* resulted in the identification of YM-60828 as a potent inhibitor of factor Xa (Ki = 1.3 nM) with selectivity over thrombin (Ki = 100,000 nM) and trypsin (Ki = 46 nM). YM-60828 has been shown to have oral activity in the squirrel monkey (20% bioavailable) (105) and found to be efficacious both by iv. and oral administration in the rat electrolytic arterial injury model of thrombosis. In this model, **17** significantly reduced the incidence of occlusive thrombus formation and improved vessel patency (106, 107).

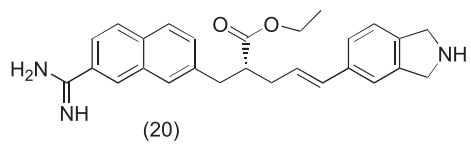
Researchers from Boehringer Mannheim have also used DX-9065a in their drug design efforts. Their approach focused on replacing the naphthamidine group with a more synthetically accessible P1 group (108). Molecular modeling studies based on the X-ray crystal structure of factor Xa led to 2-carbamimidoyl-1,2,3,4-tetrahydro-isoquinoline as the P1 replacement. Phenylacetic acid analog **18**, with pyrrolidine as the P4 group, is structurally similar to DX-9065a (108) and was found to be 2-fold less potent than the Daiichi compound. When the P4 group is replaced by N-substituted piperidines, activity generally improves. One of the most potent compounds from this study, **19**, has a Ki of 26 nM.

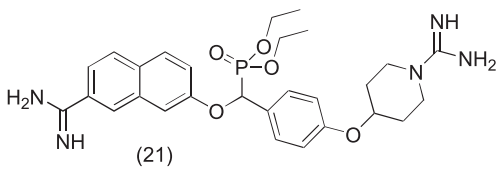
DX-9065a continues to influence many drug discovery efforts. Other inhibitors with similar structural motifs that have appeared in the patent literature are represented by **20** and **21** (109, 110).





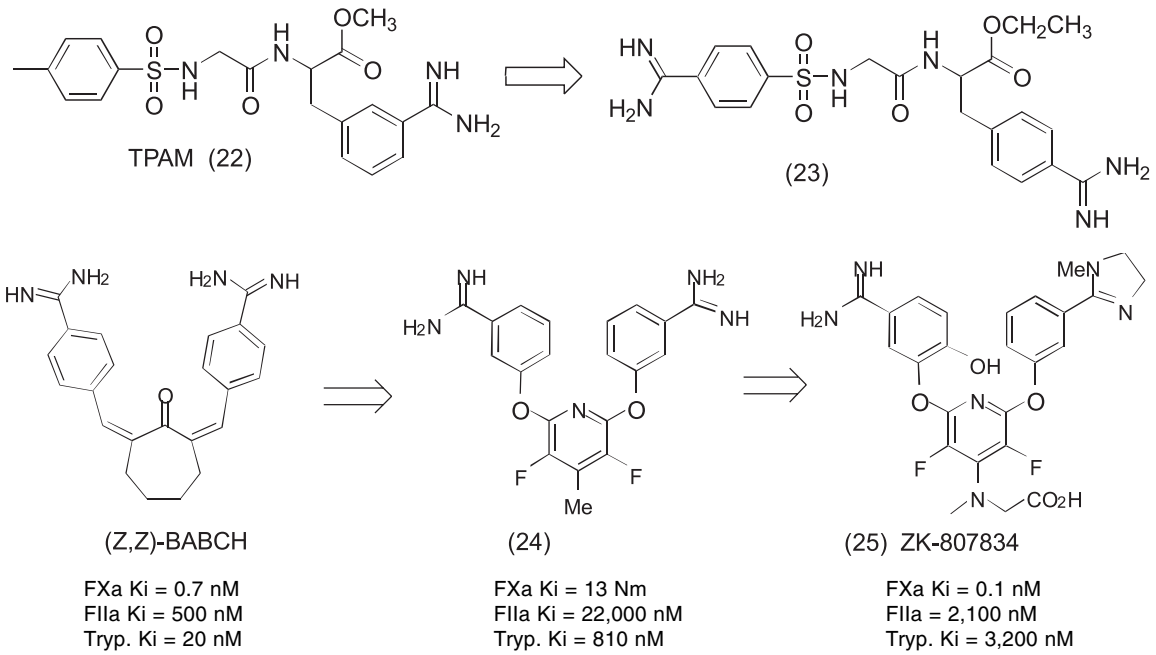






Another molecule used as a basis for inhibitor design is TPAM **22** (99). TPAM has a reported Ki for factor Xa of 1.8 μM, but has only modest selectivity for factor Xa over thrombin (Ki = 3.9 nM) and trypsin (Ki = 5.6 nM). A recently described approach to improve the profile of the ethyl ester of TPAM involved addition of an amidine to interact with the cation hole in the back of the S4 pocket (111). Examination of the X-ray crystal structure of TPAM bound in the active site of trypsin confirmed that the benzamidine group is in the S1 pocket and the tolylsulfonamide is in the S4 pocket. Hydrogen bonds are formed with Gly216 of the enzyme and the NH and carbonyl of the central glycine unit of the inhibitor. The general strategy involved replacing the methyl group of the tolyl sulfonamide with an amidine. The position of the amidine on the phenylglycine unit was varied between *meta* and *para*. One of the inhibitors arising from this study is shown below in which both benzamidines are in the *para* position. Compound **23** has a Ki of 0.5 μM for factor Xa and is selective against thrombin (Ki = 41 μM) and trypsin (Ki = 4.2 μM). Examination of the X-ray crystal structure of compound **23** bound in the active site of trypsin revealed that the amidino-phenylsulfonamide unit is in S1 and the *para*-amidino-phenylglycine unit is in S4. The reversal in binding mode was rationalized by the authors as arising from stabilization of the P1 group via H-bonding of the Ser195 hydroxyl group to one of the sulfonamide oxygens. This orientation allows for the accommodation of a *para*-benzamidme in contrast to the usual preference for *meta*-benzamidine in S1.

Berlex researchers (75, 112-114) have reported several potent factor Xa inhibitors derived from (*E,E*)-



BABCH. The first breakthrough came from the realization that the (*Z*)-isomer of BABCH was the most potent of 3 possible regioisomers (112). This observation was followed by the replacement of the cycloheptanone ring with a pyridine template and reoptimization of the P1 and P4 ligands. On this template, placement of the amidines *meta* (**24**) to the attachment points was found to be optimal.

A 4-hydroxy substituent *para* to the putative P1 benzamidine results in a 60-fold enhancement in potency attributed to the H-bonding potential of this functionality. This result was foreshadowed by the early work of Walsmann on simple derivatives, although the potency enhancement for hydroxybenzamidine *versus* benzamidine is modest (115). The P4 amidine was subsequently replaced by a methyl imidazoline moiety. Further optimization was achieved by substitution at the 4-position of the pyridine ring with sarcosine, **25**, which also increased bioavailability upon oral dosing (75).

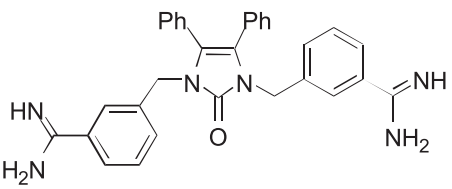
Crystallographic work has been performed on this series using trypsin as a surrogate for factor Xa. The hydroxybenzamidine is bound in the S1 pocket with the hydroxyl group forming an H-bond to Ser195 of the catalytic triad. The imidazoline is bound in the S4 pocket and the pyridine nitrogen does not appear to make an H-bond with the beta-sheet. With small variations, the trypsin bound conformations are very similar to the binding modes obtained by modeling the inhibitor in factor Xa (75).

Modifications of the central pyridine template have also been explored and potent inhibitors based on pyrimidines, purines and pteridines (116) have been described. Further variations on this theme have recently appeared in the patent literature; for example, modifications have included new pyridine substituents and/or replacement of the P4 amidine (117).

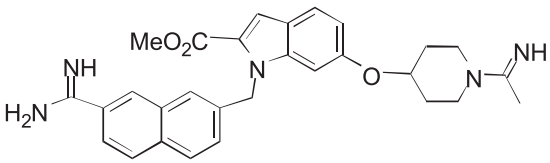
Other heterocyclic templates have been examined by the Berlex group. Bis-benzamidines such as the cyclic urea **26**, have modest activity for factor Xa (118). Another series of bis-amidines is based on indole **27** (119) and carbazole (120) templates. In an effort to increase water solubility, the original leads were ultimately superseded by the benzimidazole-based inhibitors (121). As a class, these compounds would appear to rank among the most potent small molecule inhibitors described to date. For example, compound **28** has a Ki of 0.01 nM against factor Xa. Compound **28** also exhibits significant trypsin (Ki = 3.5 nM) and factor IIa (Ki = 2 nM) activity and X-ray structures in trypsin of representative inhibitors (*i.e.,* methyl replaces isopropyl, Ki = 0.27 nM) have been obtained (121). The naphthamidine moiety is bound in the S1 pocket and the molecule adopts an extended conformation in many ways similar to the Daiichi inhibitor. Similar binding modes are found by molecular modeling studies in factor Xa (121).

Benzamidine inhibitors

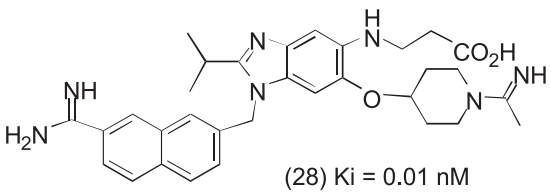
Researchers at Banyu have reported the crystal structure of FX-2212a (**29**) bound in the active site of factor Xa (122). The inhibitor is comprised of a *para*-aminopyridine unit tethered to 3'-biphenylamidine. 3'-Biphenylamidine has been identified as a P1 group that shows superior intrinsic potency and selectivity for factor Xa as compared with benzamidine (123). 3'-Biphenylamidine was found to inhibit both factor Xa (Ki = 10 μM) and trypsin (Ki = 10 μM) with selectivity over thrombin (Ki > 50 μM). The results



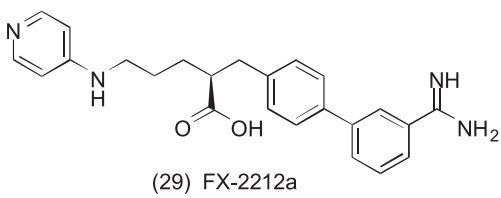
(26) Ki = 93 nM



(27) Ki = 0.17 nM



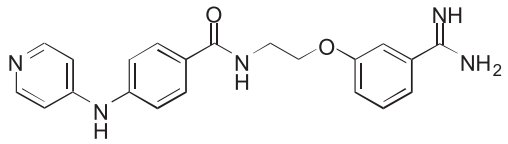
(28) Ki = 0.01 nM



(29) FX-2212a

are in contrast to benzamidine which binds weakly to factor Xa (Ki = 410 μM) as compared to trypsin (Ki = 35 μM) and thrombin (Ki = 220 μM). FX-2212a has a Ki of 0.272 μM for factor Xa as the racemate. Examination of the crystal structure revealed several key interactions. The amidine resides in the S1 pocket binding to Asp189 and the pyridine ring is in the S4 pocket with the nitrogen forming an H-bond with a structural water molecule held by Lys96 and Glu97 of the enzyme. The carboxylic acid points towards the catalytic triad forming an extended water mediated H-bond to this region. The phenyl ring adjacent to the carboxylic acid forms a hydrophobic interaction with Gln192. There are no observed H-bonding interactions of the inhibitor with Gly216 of the beta-sheet. A second group of inhibitors, reported by researchers at Ajinomoto, that also has an aminopyridine linked to a *meta*-benzamidine is represented by compound **30** (124).

The Rhône-Poulenc Rorer group has discovered a class of factor Xa inhibitors (125) in which the putative S1 (benzamidine) and S4 (biphenyl) ligands are attached by a branched hydrocarbon chain. The best compounds in this series require a *meta*-benzamidine at P1, an amide linked P4 and an ester function. Chemical correlation with



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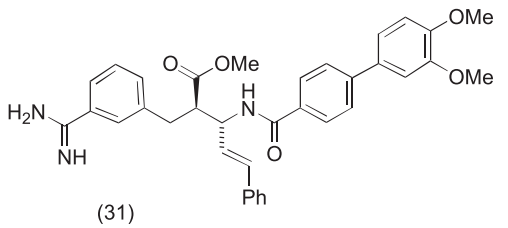


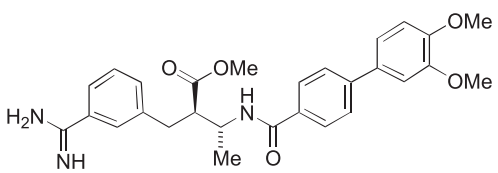
(31)

compounds of known absolute and relative stereochemistry identified the (*R,R*)-enantiomer as the most potent of 4 possible stereoisomers. Compound **31** (as the racemate) has a Ki of 10 nM for factor Xa.

A binding model consistent with these assignments has been proposed which suggests that the amide linkage provides a key hydrogen bond to Gly218 of factor Xa. The ester function is placed in close proximity to the catalytic triad residues. However, no evidence for chemical interaction with the catalytic triad has been found as these inhibitors are not hydrolyzed upon incubation with factor Xa. No obvious role for the styryl side chain is observed in the model and replacement of this group with smaller alkyl groups such as methyl yields potent inhibitors. Compound **32** (Ki = 1.3 nM) was found to be effective in the rat $FeCl_2$-induced model of thrombosis (126). Upon iv. dosing (0.5 mg/kg bolus + 0.05 mg/kg/min infusion), a 60% reduction in thrombus mass and a 3-fold prolongation in the time to occlusion as compared to controls was observed.

Based on DX-9065a and modeling studies, researchers from DuPont-Merck have also developed a linear series of bis-benzamidines (127). A branched chain was used to attach the P1 and P4 moieties. The best compounds in this series require a *meta*-benzamidine for the putative P1 ligand and a *para*-benzamidine for P4 (*e.g.,* **33**, Ki = 34 nM). As was observed in the Rhône-Poulenc Rorer series (125), the ester function was vital for optimal anti-factor Xa activity (*e.g.,* **34**, Ki = 1400 nM).

Further derivation on this series yielded *meta*-amidino-*N,N*-disubstituted anilines as potent factor Xa inhibitors (125). These analogs have the advantage of being achiral and incorporating a biphenyl moiety in place



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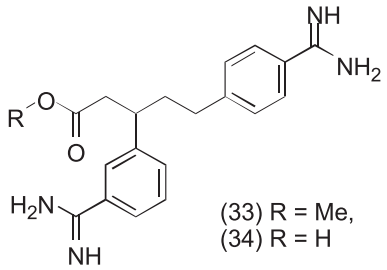
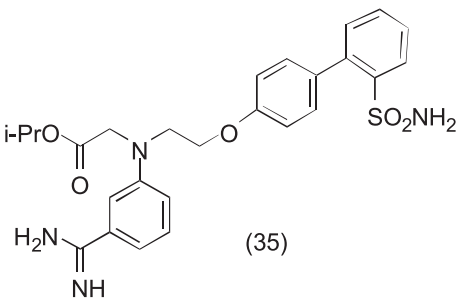


(33) R = Me,
(34) R = H



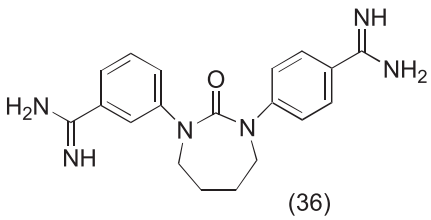
(35)

of a highly basic benzamidine group. In contrast to the carbon-based series, the achiral series is more tolerant of substitutions at the branching point. Compound **35** has a Ki = 2.1 nM for factor Xa. These inhibitors were found to have high clearance rates, reportedly due to rapid *in vivo* metabolism. It is noteworthy that highly potent compounds were obtained for the linear inhibitors of factor Xa in general, even though they lack a rigid central template.

Another series reported by the DuPont-Merck group is based on the inhibitor (*E,E*)-BABCH (129). Similar to the Berlex approach represented by compound **26**, the central cycloheptanone is replaced by a 7-membered cyclic urea. A number of positional isomers around the amidines were examined with compound **36** having the best activity (Ki = 800 nM). Replacement of one of the amidines with a benzylpiperidine unit results in compound **37**, which is 8-fold more potent (Ki = 100 nM) than the bis-benzamidine **36**.

Isoxazoline factor Xa inhibitors have been described by the DuPont-Merck group (130). The initial screening lead was modified to the bis-benzamidine **38** (Ki = 870 nM). Substitutions on the isoxazoline ring led to rapid improvements in potency; the methyl acetate derivative, **39**, was found to have a Ki of 94 nM. For this series, the combination of *meta*-benzamidine in S1 and *para*-benzamidine in S4 resulted in the best potency. Interestingly, when the relative orientation of the amidines was switched **40**, the resulting analog was still active with a Ki of 117 nM.

The subject of other studies in this series focused on nonbasic P4 groups (131, 132). The DuPont group has identified the 2'-sulfonamidobiphenyl moiety in their development of thrombin inhibitors (133). Application of the biphenyl group to their series of factor Xa inhibitors resulted in potent derivatives. SAR studies have been presented in which the isoxazolines and isoxazoles have been modified by appending side chains and changing the position of the amide linker (133). In the first class



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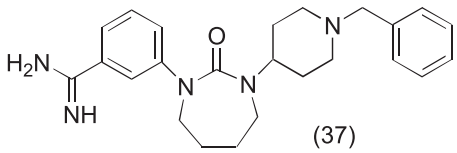

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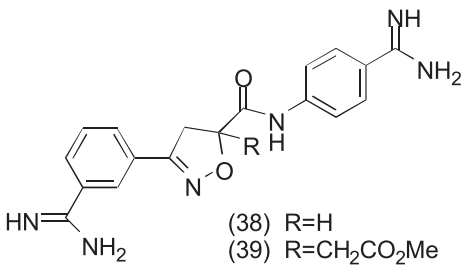
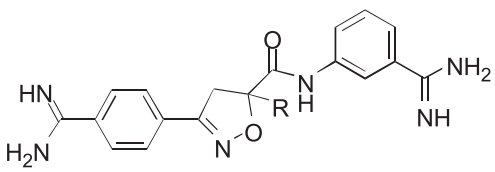


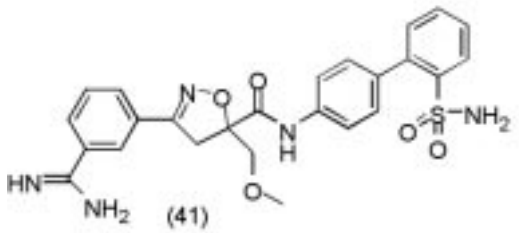
(38) R=H
(39) R=CH_2CO_2Me



(40) R=CH_2CO_2Me

shown below, with both the methoxymethyl side chain and the amide originating from the 5-position, several inhibitors with Ki values of < 2 nM were identified. Compound **41** is highly selective for factor Xa (Ki = 0.55 nM) over thrombin (Ki = 4600 nM) and trypsin (Ki = 109 nM). Transposing the side chain bearing the biphenyl group to the 4-position gives inhibitor **42** with no loss in potency (Ki = 0.34 nM) and with a similar selectivity profile. The achiral isoxazole, compound **43**, again gave a similar potency and selectivity for the case where R is methoxymethyl. Interestingly, for the achiral isoxazole, removal of the methoxymethyl side chain results in a highly potent compound **44**. which has a Ki of 0.15 nM for factor Xa.

The Rhône-Poulenc Rorer group developed a series of inhibitors based on a *de novo* design approach (134, 135) using the X-ray crystal structure of factor Xa (65). The first series arising from this approach used 1,3-disubstituted indole as a template to which are appended the P1 and P4 groups. Modestly potent compounds (Ki = 900 nM for **45**) could be obtained with the indole scaffold. Alternate templates were explored in an effort to improve solubility properties and to introduce functionality that could H-bond with the β-sheet of the factor Xa active site. This study resulted in the identification of 3-amino-



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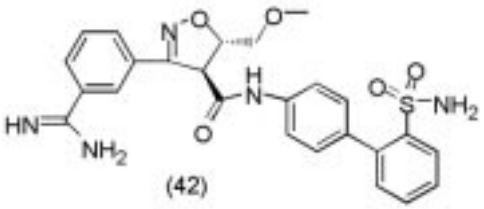

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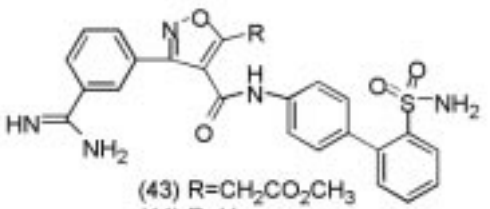
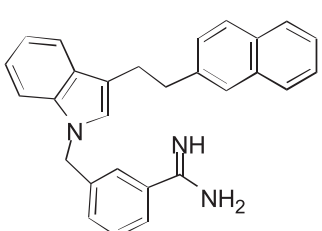


(43) R=$CH_2CO_2CH_3$
(44) R=H

2-pyrrolidinone as a central template that gave potent and selective factor Xa inhibitors (136). Compound **46** has a Ki of 230 nM for factor Xa with selectivity over thrombin (Ki = > 4000 nM) and trypsin (Ki = 2900 nM).

Initial optimization studies led to **47**, in which the P4 group is 7-methoxynaphthalene and P1 is a thiophene amidine. This inhibitor has a Ki of 7 nM for factor Xa with selectivity against thrombin (Ki = 1000 nM) and trypsin (Ki = 530 nM). Compound **47** is efficacious in both a rat model of venous thrombosis and in a rabbit model of arterial thrombosis (137). Further potency enhancements of the pyrrolidinone series was achieved by the incorporation of 4-substituted-benzamidines (115) and basic P4 groups designed to interact with the S4 cation hole (138). These studies yielded compound **48** as a potent inhibitor of factor Xa (Ki = 2 nM) with three orders of magnitude selectivity over a number of serine proteinases including thrombin, trypsin, plasmin, activated protein C, tissue plasminogen activator and factor VIIa. Compound **48** was found to be effective in the rat $FeCl_2$-induced model of thrombosis. Upon i.v. dosing (0.3 mg/kg bolus + 0.03 mg/kg/min infusion) compound **48** displayed a 50% reduction in thrombus mass and a 3-fold prolongation in



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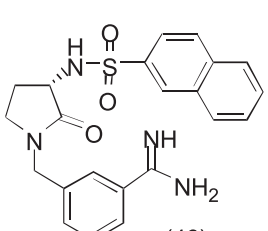

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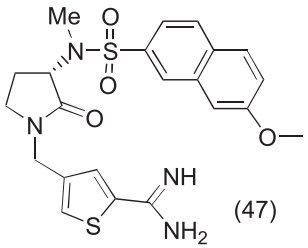

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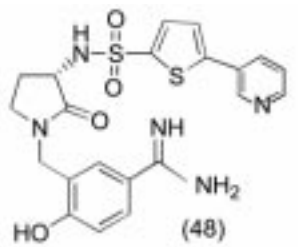

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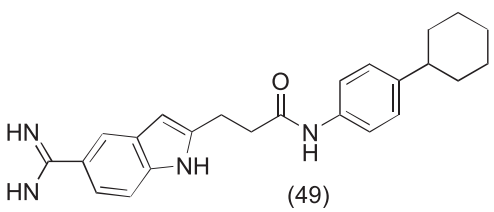

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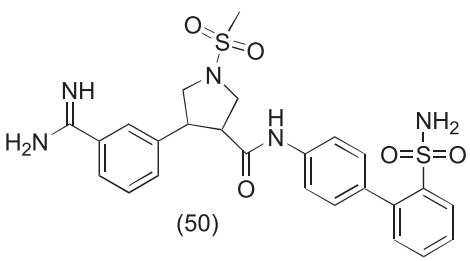


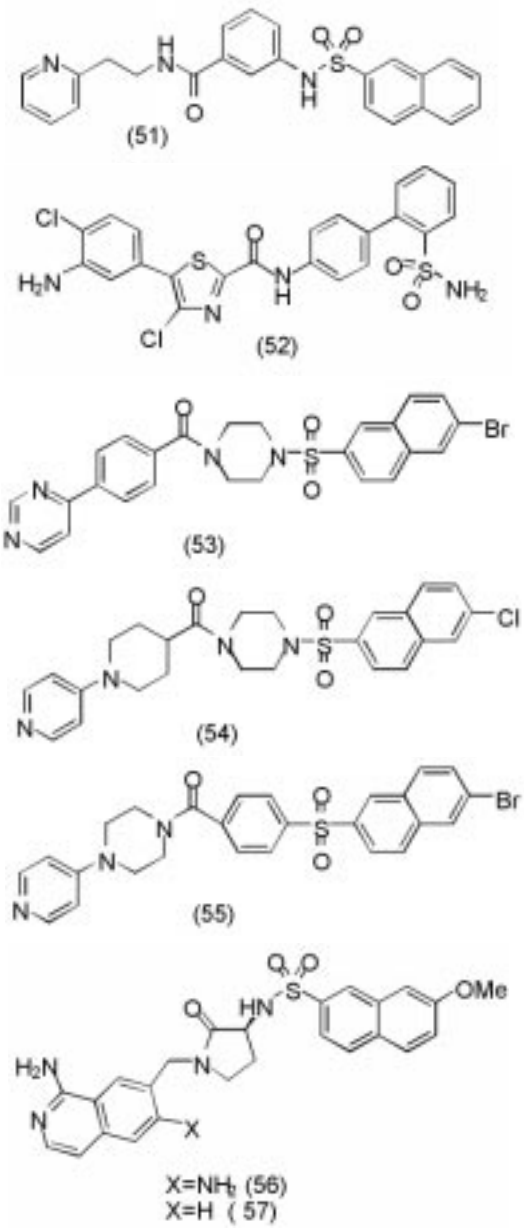
(50)

the time to occlusion as compared to controls. No effects on heart rate or blood pressure were observed for these compounds as is often seen with amidine containing compounds (139).

Benzamidine inhibitors containing putative nonbasic P4 groups continue to appear in the patent literature. Compounds **49** and **50** are two representative examples from researchers at DuPont-Merck (140, 141).

Nonbenzamidine inhibitors

Factor Xa inhibitors which do not contain amidines or other highly basic functions have appeared in the the patent literature. Of these, the piperazines, **53-55**, described by Zeneca are of particular interest since nanomolar potencies are claimed for certain members of this class (142-144). For example, compound **54** has an IC_{50} of 3 nM for factor Xa with high selectivity for factor Xa over thrombin (IC_{50} = > 34,000 nM). Other patented non-benzamidine Xa inhibitors include compound **51** from



3D-Pharmaceuticals and compound **52** from DuPont-Merck (146, 147).

Researchers at Rhône-Poulenc Rorer have discovered a class of factor Xa inhibitors wherein the basic P1 of the earlier pyrrolidinone inhibitors was replaced by a benzamidine isostere, namely an aminoisoquinoline (148). This change resulted in a pKa decrease of 4 log units as compared to benzamidine. A representative inhibitor **56** has a Ki of 80 nM against factor Xa and is selective against trypsin (Ki > 2900 nM). Modeling studies with compound **57** in factor Xa indicate that the aminoisoquinoline occupies the S1 subsite while the methoxynaphthalene group fills the S4 pocket. The isoquinoline ring makes extensive hydrophobic contacts with residues in S1 while the amino group forms H-bonds with Asp189. The discovery of factor Xa inhibitors which lack highly basic functions (*i.e.,* amidines) holds considerable promise for future design since similar advances in the thrombin inhibitor field is what ultimately led to the discovery of orally effective factor IIa inhibitors (58).

Conclusions

Using modeling techniques and X-ray analysis, discovery efforts have been rapidly departing from the symmetrical bis-benzamidine motif and peptidyl structures found in early inhibitors of factor Xa. Many of the recently reported approaches involve reducing the overall basicity of the inhibitors and series of compounds with one benzamidine are appearing with more frequency. The design of nonbenzamidine inhibitors is an emerging area of research, with novel structures beginning to appear in the patent literature. Improvement in pharmacokinetic and pharmacodynamic properties are anticipated from inhibitors with reduced basicity, especially in light of the progress made in the area of thrombin inhibition.

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