DOI: 10.1002/anie.201004575

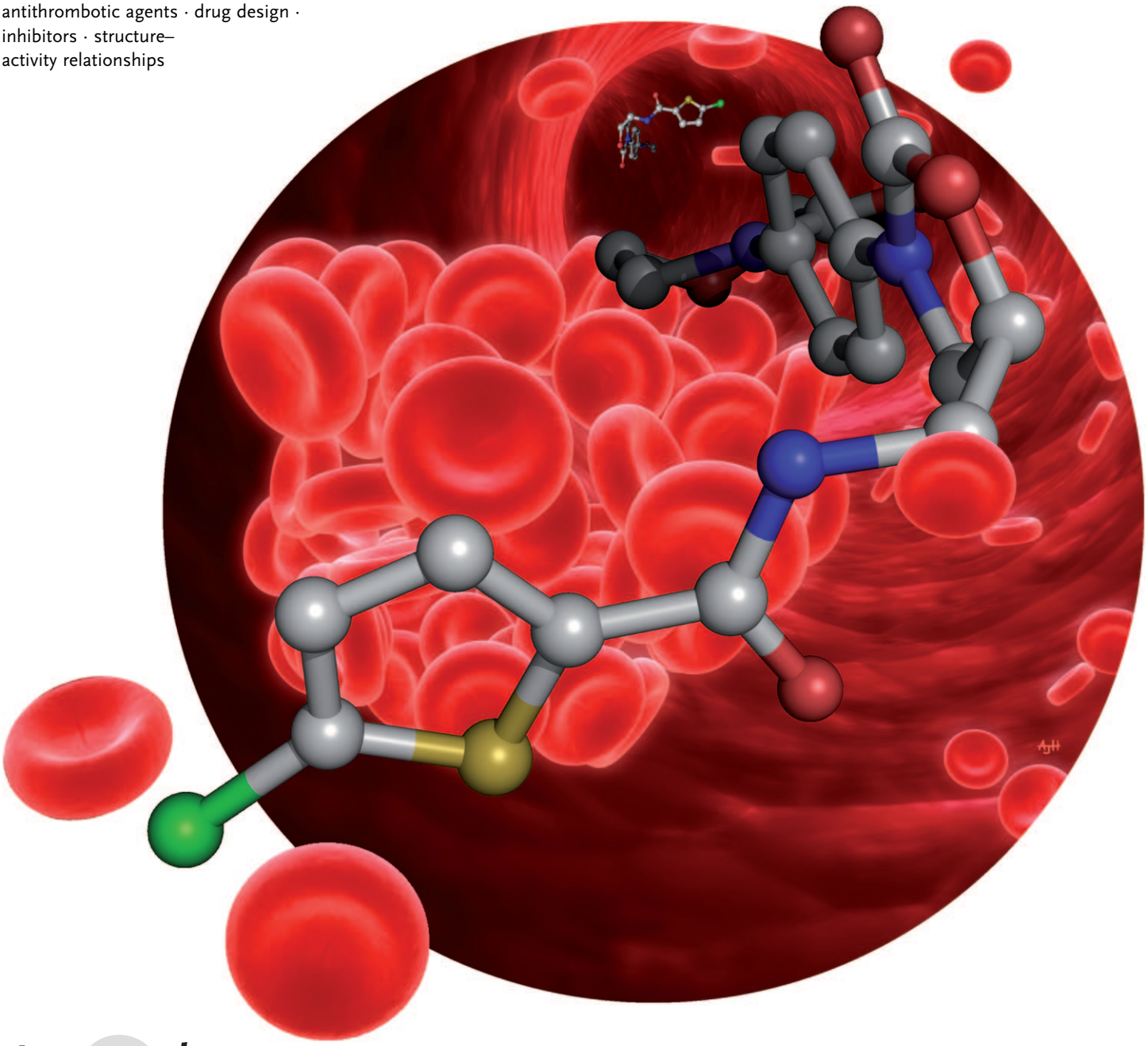
Protease Inhibitors

Oral, Direct Thrombin and Factor Xa Inhibitors: The Replacement for Warfarin, Leeches, and Pig Intestines?

Alexander Straub, Susanne Roehrig, and Alexander Hillisch*

Keywords:
antithrombotic agents · drug design · inhibitors · structure–activity relationships

Dedicated to Professor Franz Effenberger



To prevent thromboses after surgery, patients have until now had to inject themselves daily with heparin. For stroke prophylaxis in atrial fibrillation, patients take vitamin K antagonists of the coumarin type, which have a narrow therapeutic window and whose dosage must be regularly monitored. In order to improve the standard of therapy in thromboembolic diseases such as deep-vein thrombosis, pulmonary embolism, and stroke in atrial fibrillation, intensive research has been carried out over the last decade in the search for new, orally active thrombin and factor Xa inhibitors. A number of these compounds are already on the market or are in advanced clinical development; they could revolutionize the anticoagulant market.

From the Contents

1. Introduction

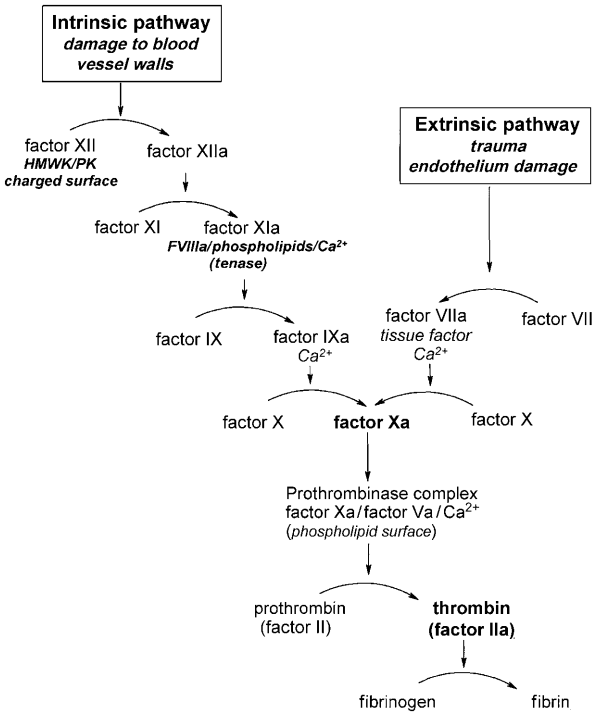
Hemostasis is a complex mechanism which protects the organism from life-threatening blood loss after tissue damage. It is activated by two pathways:

In primary (cellular) hemostasis following arterial vessel wall damage, which can also have arteriosclerotic causes, the Von Willebrand factor is released; it binds to thrombocytes and can thus initiate thrombocyte aggregation and thrombus formation. This course can be interrupted by the use of thrombocyte aggregation inhibitors. For example, P2Y12 receptor antagonists such as clopidogrel, and cyclooxygenase inhibitors such as acetylsalicylic acid (ASA), which inhibit the formation of proaggregatory thromboxane A2, are used for this purpose.

Secondary plasmatic hemostasis triggers the blood-clotting cascade (Scheme 1) with the aim of stabilizing platelet aggregates by fibrin cross-linking. It also comes into effect especially when there is reduced blood flow as a result of surgery, immobilization, or atrial fibrillation. Resulting shear forces and the predominance of hypoxic conditions lead to the formation of tissue factors.

The intrinsic contact activation pathway begins with the formation of a primary complex from HMWK (high-molecular-weight kininogen), prekallikrein (PK), and factor XII (FXII). By interaction with collagen or other negatively charged surfaces (e.g. in external injury at the vascular endothelium, lipoproteins in hyperlipidemia, bacteria in infections) FXII is activated to FXIIa. FXIIa cleaves FXI to FXIa which, in turn complexed with FVIIIa and phospholipids (tenase complex), converts FIX into FIXa and this activates FX into FXa.

The extrinsic pathway (tissue factor pathway) is activated in an injury without direct contact with nonphysiological surfaces, by endothelium damage, or by hypoxia resulting from reduced bloodflow.[1] In this situation tissue factor (TF, thromboplastin, FIII), a surface membrane glycoprotein, is presented from the damaged tissue and binds circulating factor VIIa and VII, which facilitates the transformation of FVII into FVIIa. The complex from FVIIa, TF, calcium ions, and phospholipids catalyzes the conversion of FX into FXa. It has been shown that the plasma concentration of TF is also elevated in atrial fibrillation.[2]



Scheme 1. The blood-clotting cascade.

Thus intrinsic and extrinsic pathways converge at factor Xa (FXa). In its free form FXa is only weakly active. It is strongly activated by the formation of the prothrombinase complex from FXa, cofactor Va, and calcium ions on phospholipid surfaces of thrombocytes. In this form it cleaves prothrombin to thrombin. This step means an approximately

[*] Dr. A. Straub, Dr. S. Roehrig, Prof. Dr. A. Hillisch
Bayer HealthCare Pharmaceuticals, Medicinal Chemistry Wuppertal
42096 Wuppertal (Germany)
E-mail: alexander.straub@bayer.com

1000-fold enhancement. Thrombin cleaves off the fibrinopeptides A and B from fibrinogen, whereby the fibrin monomers thus formed are cross-linked by FXIII (a transglutaminase) likewise activated by thrombin.

Thrombin stimulates the activation of the clotting factors FXI, FIX, FX, and FXIII and the cofactors FV and FVIII, and in this way amplifies its own formation. Higher concentrations of thrombin together with thrombomodulin activate the thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI cleaves off the C-terminal Lys and Arg residues from fibrin. Since this is the docking site for fibrinolytic plasminogen, the ternary complex of plasminogen and tPA (tissue plasminogen activator) can no longer form on the fibrin surface. This leads to downregulation of plasminogen activation and consequently of fibrinolysis. Thrombin also activates the protease-activatable receptors (PAR) on the surface of blood platelets, which are hence stimulated towards aggregation, thus providing the second important component of blood clotting. Thrombin initiates proliferation and migration processes on vascular smooth muscle cells which are important for arteriosclerotic processes.

Excess thrombin that circulates outside the region of the lesion is bound on intact endothelial cell surfaces by the membrane protein thrombomodulin. The resulting complex has no further proclotting activity but instead causes activation of the serine protease protein C. Together with the cofactor protein S, activated protein C has clotting inhibitory properties in which it degrades the phospholipid-bound factors Va and VIIIa. In this way the action of thrombin is restricted to the region of the vessel injury, the activated clotting system is moderated, and a systemic reaction is avoided.

Therefore, different pharmacological profiles for FXa and thrombin inhibitors are to be expected. Whereas with FXa inhibition the de novo synthesis of new thrombin is inhibited, circulating thrombin can only be influenced by thrombin inhibitors.

Thrombin and factor Xa are inhibited in the body by antithrombin (AT). Heparin, a sulfated glycosaminoglycan isolated industrially from porcine intestine or bovine lung, is a strong allosteric activator of AT. The actual physiological function of heparin is supposed to lie in the defense of mast cells against bacteria. By fragmentation and fractionation low-molecular-weight heparins (LMWH) such as enoxaparin can be obtained from heparin, which have more favorable pharmacokinetic properties, cause fewer adverse effects, and have a more selective action against FXa. Today these intravenously or subcutaneously administered drugs are used routinely in the clinic, but they still are of animal origin and thus can be contaminated. A fully synthetic pentasaccharide partial sequence of heparin stabilized as the methylglycoside in complex with AT inhibits only factor Xa, has a long half-life, and is used in the clinic as the drug fondaparinux after larger orthopedic surgery.[3] The prothrombinase complex itself is, however, no longer accessible to inhibition by the AT-bound indirect FXa inhibitor.[4] Direct FXa inhibitors have the additional advantage of also inhibiting the prothrombinase complex.

Indirect (i.e. mediated by AT) FXa inhibitors are not absorbed orally and must therefore be injected subcutaneously. Whereas this may still be acceptable for a short time in hospital, for a required longer-term therapy such as stroke prophylaxis in atrial fibrillation the patient must have access to an oral anticoagulant.

For this purpose, vitamin K antagonists such as warfarin and other coumarin derivatives are currently used. Vitamin K participates as a cofactor in the glutamate carboxylation of clotting factors II, VII, IX, and X. The calcium-binding property of the resulting malonyl unit possessing the additional carboxylate group is essential for biological activity of these factors.

Coumarin derivatives suffer from a narrow therapeutic window, which is caused by genetic polymorphism of the metabolizing enzymes and by the covalent binding of the coumarins to vitamin K epoxide reductase. Significant costs arise with this therapy owing to adverse effects (hemorrhages) and the necessary close monitoring of the clotting parameters in the clinic.

Thromboembolic diseases such as myocardial infarction, stroke, pulmonary embolism, deep-vein thrombosis, and peripheral arterial occlusive diseases are still amongst the most frequent causes of death. Consequently there is a considerable need for direct acting, oral anticoagulants with a broader therapeutic window and reliable efficacy.

Alexander Straub, born 1959, studied chemistry in Stuttgart, obtained his doctorate with Prof. F. Effenberger, and then joined the pharmaceutical research division of Bayer AG as a medicinal chemist. After ten years he moved to Bayer CropScience where he worked on chemical production processes in the department for process research and process development for eight years. Since 2008 he has been working as a principal research scientist on cardiovascular indications at Bayer HealthCare Pharmaceuticals. He is a co-inventor of the factor Xa inhibitor rivaroxaban.

Susanne Roehrig studied chemistry at the Ruhr University Bochum and she obtained her doctorate in 1997 with Prof. P. Welzel at the University of Leipzig. After postdoctoral research at the Massachusetts Institute of Technology in the group of Prof. P. H. Seeberger, she moved to the Institute for Medicinal Chemistry of Bayer HealthCare Pharmaceuticals and has worked there since then in the area of cardiovascular indications. She is a co-inventor of the factor Xa inhibitor rivaroxaban.

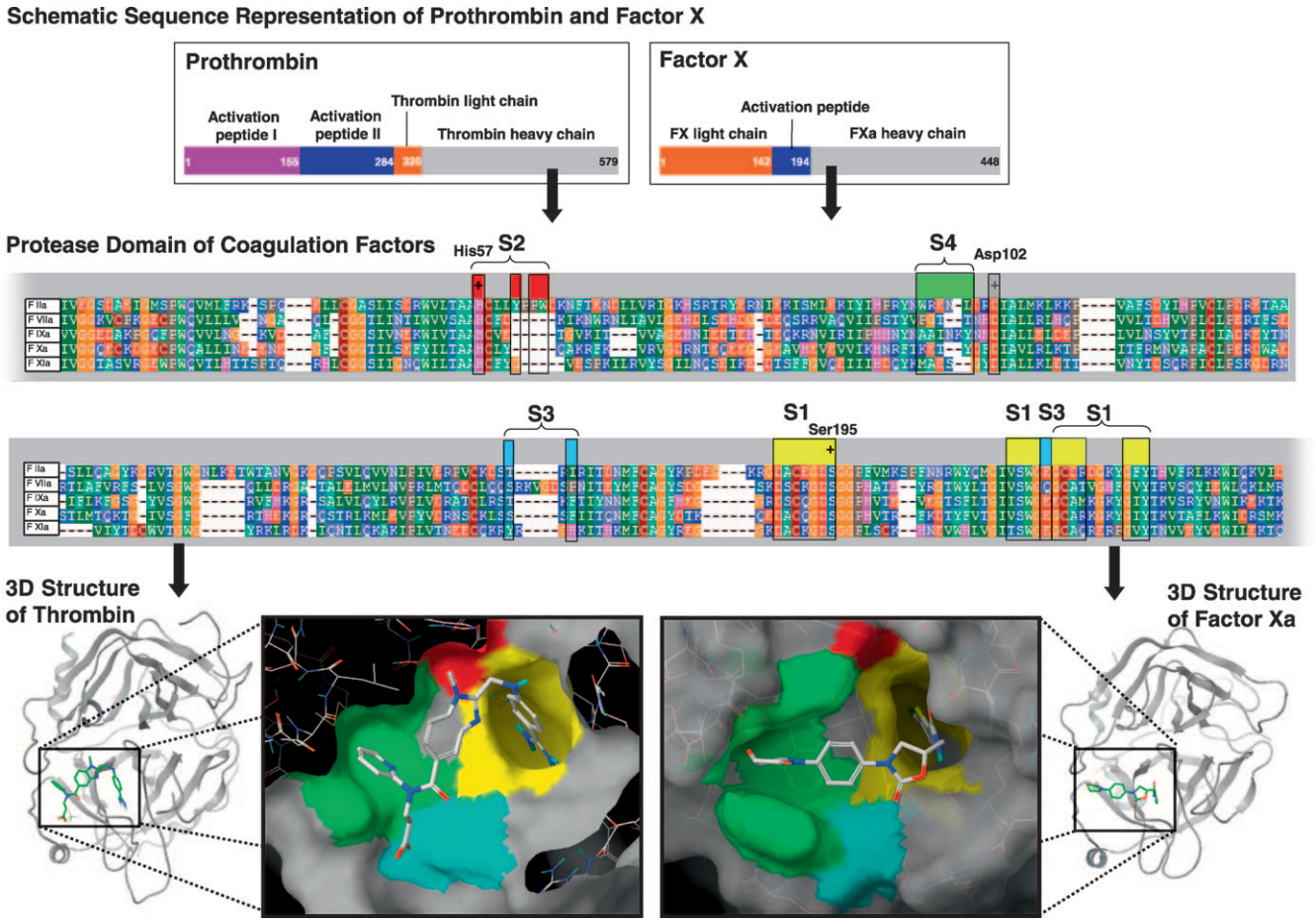


Figure 1. Scheme of the total sequence of prothrombin and FX and their cleavage sites and the alignment of sequences (middle) of thrombin (FIIa), FVIIa, FIXa, FXa and FXIa. The heavy chains containing the protease domain are labeled in gray. The substrate binding pockets S1 (yellow), S2 (red), S3 (blue), and S4 (green) as well as the amino acids involved in the catalytic mechanism (His57, Asp102, Ser195) are highlighted. Bottom: Comparison of the substrate binding sites of thrombin with dabigatran (left) and FXa with rivaroxaban (right). The coloring of the surfaces corresponds to that given above. Colors in the stick models of the inhibitors: white/green C, cyan H, blue N, red O, yellow S.

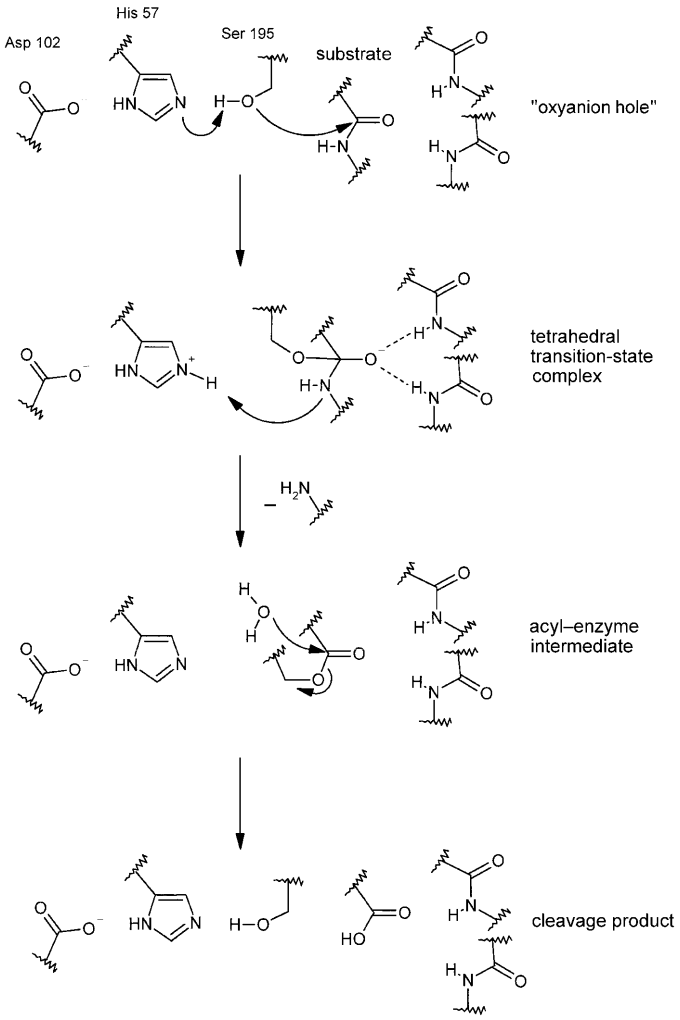
2. Structural Comparison and Substrate Properties of Thrombin and Factor Xa

FXa consists of a trypsin-like serine protease composed of 254 amino acids and a light chain (142 amino acids), which is built up of a glutamate-rich "Gla" domain and two epidermal growth factor (EGF)-like domains. FXa is formed from the precursor protein FX, from which a 52 amino acid activation peptide is cleaved off either by FIXa or by FVIIa.

α-Thrombin is a protein with a molecular mass of 36 kDa; it consists of the 36 amino acid light chain and the trypsin-like peptidase domain (259 amino acids), also called the heavy chain. The enzyme FXa cleaves the activation peptides 1 and 2, consisting of 155 and 129 amino acids, respectively, from the prothrombin, which consists of 579 amino acids. Subsequently, separation of the light from the heavy chain of thrombin takes place, whereby both strands remain connected covalently through a disulfide bridge.

The two serine protease domains of thrombin and FXa share a sequence identity of 37 % (Figure 1). In both enzymes, the catalytic center is formed from the triad His57, Asp102, and Ser195 (the numbering of the amino acids refers to the historically earlier characterized bovine chymotrypsinogen). The negatively charged tetrahedral transition state at the carbonyl group of the substrate, which is formed during the amide cleavage by covalent binding to the serine, is stabilized by the so-called "oxyanion hole" by hydrogen bonds with amino acids of the enzyme (Scheme 2). According to the nomenclature of Schechter and Berger, the amino acids of the

Alexander Hillisch, born in 1971, studied pharmacy at the University of Vienna and then moved to the Institute of Molecular Biotechnology in Jena. He gained his doctorate in biochemistry with Prof. P. Schuster in 1998 and moved to EnTec GmbH Hamburg/Jena. There he built up a research group for structural bioinformatics and drug design and led a number of drug discovery projects. In 2003 he moved to Wuppertal as section head for computational chemistry in the medicinal chemistry department of Bayer HealthCare Pharmaceuticals. In 2010 he was appointed honorary professor by the University of Cologne.



Scheme 2. Catalysis mechanism of serine proteases.

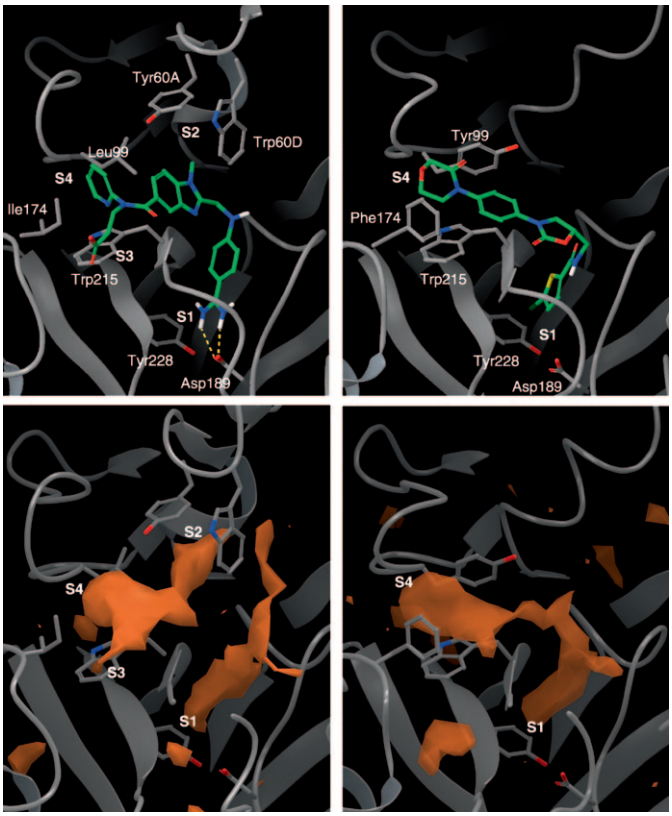


Figure 2. Comparison of the active center in the crystal structures of thrombin and factor Xa. Top left: thrombin with dabigatran (**9**; modeled on the basis of the structure with the PDB code 1KTS); top right: factor Xa with rivaroxaban (**34**; crystal structure: PDB code 2W26). Bottom: lipophilic impression of the binding pocket (orange volume), prepared by probing the binding pocket with lipophilic probe atoms; bottom left: thrombin; bottom right: FXa.

substrate from the N terminus to the cleavage site are termed P4-P3-P2-P1 and the corresponding binding pockets of the enzyme, S4-S3-S2-S1.

At the bottom of the S1 specificity pocket of both enzymes Asp189 is located, which participates in ionic and hydrogen-bond interactions with the positively charged P1 arginine of the substrate (Figure 2). According to the sequence alignment, thrombin and FXa have the same amino acids in the specificity pocket (Figure 1). They are highly similar in their three-dimensional structures in this pocket also.

In contrast the differences in the size of the S2 pocket of thrombin and FXa lead to the preference for different substrates: because of the large side-chain Tyr99 FXa has a very small S2 pocket and preferentially cleaves substrates with smaller amino acids at P2 (e.g. glycine). Two exemplary cleavage sites (-//-) are the sequences Phe281-Asn282-Pro283-Arg284-//-Thr285-Phe286 and Tyr317-Asp318-Gly319-Arg320-//-Ile321-Val322 from human prothrombin. FXa cleaves between Arg284 and Thr285 and between Arg320 and Ile321 (P1/P1′), which leads to the separation of the light chain from the activation peptide 2 and the heavy chain and hence the activation of thrombin. Thrombin contains an additional "60-insertion loop" (named after Trp60D) consisting of nine amino acids which forms a relatively large lipophilic S2 pocket. As a result, this enzyme prefers larger amino acids in the P2 position (e.g. Val, Pro). Two exemplary cleavage sites (-//-) for thrombin are the sequences Gly13-Gly14-Val15-Arg16-//-Gly17-Pro18 and Phe11-Ser12-Ala13-Arg14-//-Gly15-His16 from the fibrinogen-α and fibrinogen-β chains. Thrombin cleaves between Arg16 and Gly17, and between Arg14 and Gly15 (P1/P1′), which leads to the release of the 16 and 14 amino acid long fibrinopeptides A and B, respectively, as well as to the start of the polymerization of the resulting fibrin.

In both enzymes the S3 pockets are relatively flat. The term "pocket" is at this point somewhat misleading since the protein surfaces do not form any noticeable cavity in this region. An "imprint" of the ligand binding pocket calculated theoretically with lipophilic probe atoms shows overall significantly larger lipophilic contacts in the thrombin pocket than in the FXa pocket (see orange volume in Figure 2).

Differences in the S4 pocket are more pronounced. In thrombin, this pocket is formed by two aliphatic lipophilic amino acids (Leu99 and Ile174) and the aromatic Trp215 at

the bottom. In Fxa, through the lateral lining of aromatic amino acids (Try99, Phe74), it is very deep and hydrophobic. In this enzyme there is an ensemble of several carbonyl groups of the protein backbone located in the region of the S4 pocket, the so-called "cation hole". Hence hydrophobic interactions as well as electrostatic interactions with positively charge inhibitors are possible.

3. Thrombin Inhibitors

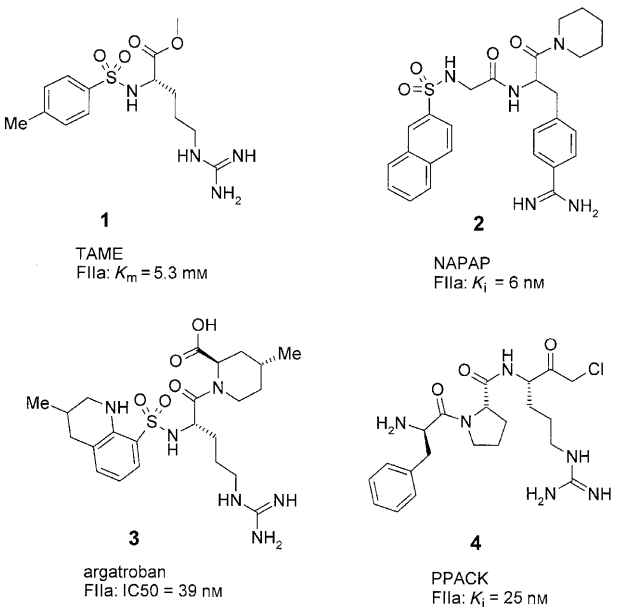
The best characterized, naturally occurring thrombin inhibitor is the polypeptide hirudin (65 amino acids)[5] from the medicinal leech (*Hirudo medicinalis*) which was isolated for the first time in 1955 by the pharmacologist Fritz Markwardt[6] by extraction from leech heads. Hirudin is a bivalent, highly selective, and essentially irreversible inhibitor ($K_i = 22$ fM), which at the same time binds to the fibrinogen binding site of thrombin and inhibits the active center of thrombin through an extensor. Today, hirudin is produced by genetic recombinant techniques.

Initial in vivo investigations[7] with hirudin at the beginning of the 1980s showed that specific thrombin inhibitors have considerable potential in the development of novel anticoagulants. Subsequently, numerous research groups initiated programs for the development of synthetic inhibitors, during the course of which several strategies were followed.

Firstly, bivalent inhibitors were developed in analogy to hirudin. Hirulog,[8] a synthetic analogue of hirudin with 20 amino acids, is a direct, bivalent reversible thrombin inhibitor with a short elimination half-life of approximately 25 minutes and is marketed under the name bivalirudin (bivalent hirudin, Angiox). Bivalirudin inhibits thrombin by binding to the active site and to the fibrinogen binding site. This inhibition is reversible since thrombin cleaves bivalirudin, and the two fragments have only a low affinity for thrombin. In patients, the action therefore subsides rapidly: 80% of the bivalirudin is lost by proteolysis, while 20% is eliminated intact through the kidneys.

The majority of the research programs have, however, targeted monovalent inhibitors which block only the active site of thrombin. The primary cleavage site for thrombin in its substrate fibrinogen lies between the amino acids Gly and Arg, and for this reason, the first potent direct thrombin inhibitors (DTIs) were also basic arginine derivatives (Scheme 3). The first prototypes of this subclass, NAPAP (**2**)[9] from the group of Stürzebecher and argatroban (**3**)[10] from the group of Mitsubishi, are both derived from the arginine derivative TAME (**1**)[11], which itself is only weakly active.

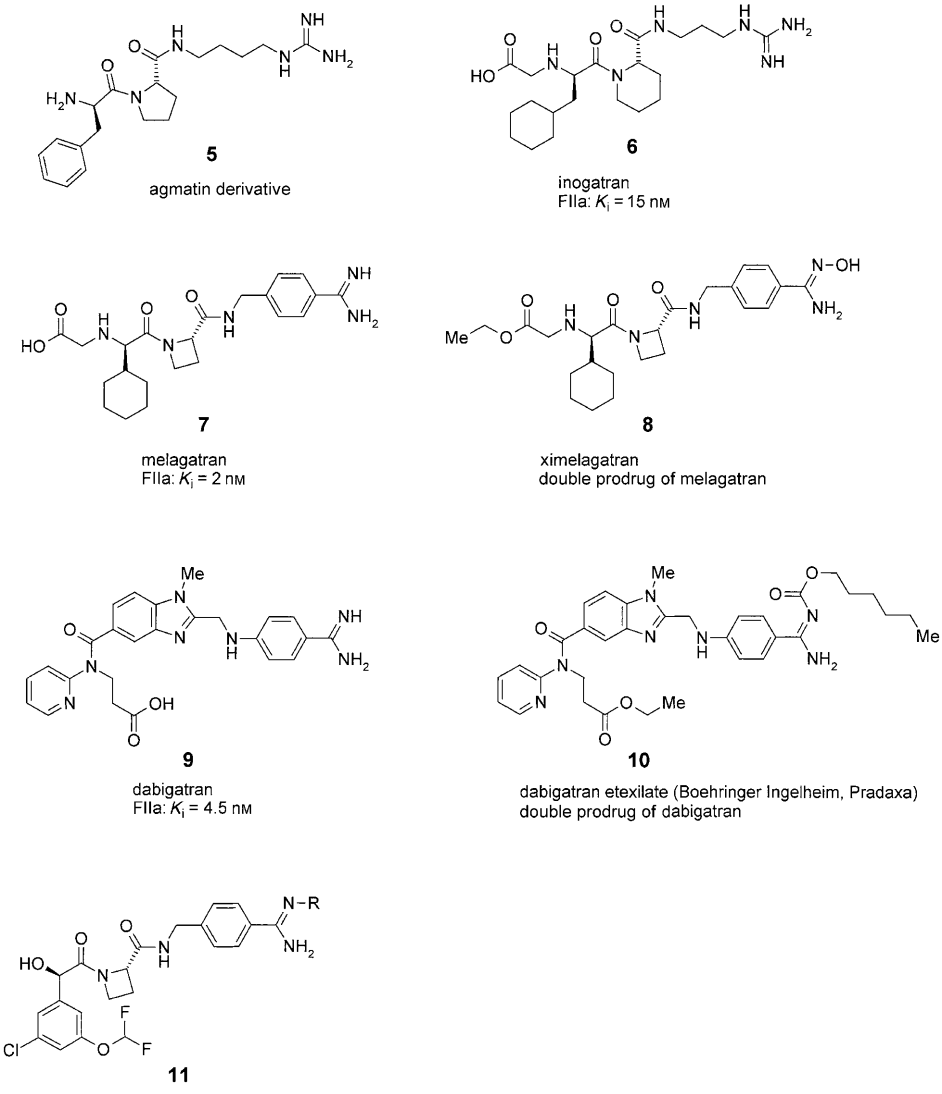
According to their crystal structures,[12] both compounds are monovalent, direct thrombin inhibitors that bind selectively to the active site of thrombin. The respective arylsulfonyl unit occupies the hydrophobic S4 pocket, and the basic amidinyl or guanidinyl unit enters into a direct electrostatic interaction with the amino acid Asp189 at the bottom of the S1 pocket.[13] However, owing to this highly basic P1 residue, neither compound is orally bioavailable. Argatroban (**3**) is the only representative of this first generation that has undergone clinical development; it was authorized for marketing for the first time in 1990 in Japan for parenteral antithrombotic therapy (Argatra).



Scheme 3. Basic thrombin inhibitors.

Another class of thrombin inhibitors contains the tripeptide motif D-Phe-Pro-Arg. This motif is derived from the amino acids involved in the binding mode of fibrinopeptide A, an inhibitory acting product of the thrombin-catalyzed cleavage of fibrinogen. The peptide D-phenylalanyl-L-prolyl-L-arginine-chloromethylketone **4** (PPACK) inhibits thrombin very potently as an irreversible inhibitor.[14] In 1989, the crystal structure of the complex of human thrombin with PPACK (**4**) was published by Bode and co-workers.[15] Here, too, the basic side chain of the P1 arginyl residue enters into an electrostatic interaction with the free carboxyl group of Asp189 in the S1 pocket. The pyrrolidine ring of the proline and the phenyl residue of the D-phenylalanine are located in the two hydrophobic binding pockets S2 and S4 of thrombin. The chloromethylketone function of the arginyl residue binds covalently to the hydroxy group of Ser195 forming a hemiketal and alkylates the imidazole nitrogen atom of His57 in the active site. Modification of this amino acid residue leads to total loss of the catalytic activity.

Polypeptide structures do indeed allow many interactions with thrombin and thus result in potent inhibitors. However, only inhibitors with small molecular masses ($< 500\ g\,mol^{-1}$) can readily pass through the gastrointestinal epithelium and enter the blood circulation. Therefore, the dipeptide structures published by Bajusz et al. in 1982 such as D-Phe-Pro-Agmatin (**5**),[16] although only weakly active, became the important starting point for further research work (Scheme 4). AstraZeneca was the first to successfully pursue this dipeptide approach and starting from **5** developed the clinical candidate inogatran (**6**), which is 100 times more potent in vitro but which has inadequate bioavailability and is excreted relatively rapidly.



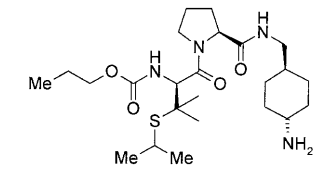
Scheme 4. Genesis of thrombin inhibitors and their prodrugs.

After elucidation of the X-ray crystal structure of the complex[15] of human thrombin with the irreversibly bound inhibitor PPACK (**4**) at the end of the 1980s, researchers at AstraZeneca profiled more than 100 potential inhibitors in their computer model and came to the clinical candidate melagatran (**7**), which is eight times more potent than inogatran (**6**). The initial clinical studies revealed that **7**, with an oral bioavailability of only 3–7%, is not suitable for oral therapy, particularly as unpredictable, variable plasma concentrations and a pronounced food effect were observed.[17,18] With two basic groups (benzamidinyl and secondary amino groups) and an acidic carboxylic acid group, at intestinal pH, **7** is protonated and overall still a positively charged compound. Thus it can penetrate the lipophilic gastrointestinal wall only to a modest extent. It was found possible to convert melagatran into a more lipophilic double prodrug (**8**, ximelagatran, Exanta), which after successful absorption is rapidly metabolized to the active substance melagatran. Compound **8** was the first oral thrombin inhibitor to make its way into the clinic. It is 170 times more lipophilic than melagatran (**7**) and exists practically uncharged at physiological pH values; this results in an 80 times improved absorption and thus in a fourfold improved oral bioavailability of 18–24%. The reasons are that the hydroxyamidine is less basic than the amidine and that the pK_a value of the N-terminal secondary amine is lower owing to the ethyl ester group. Ximelagatran was approved for marketing in Europe in 2004 but was withdrawn from the market again in 2006 after the FDA refused approval because of problems with liver toxicity.

Nevertheless, the development of other DTIs was pursued further, and in 2008 Boehringer Ingelheim was able to bring the first DTI to the market with dabigatran etexilate[19] (**10**, Pradaxa). This drug was also designed by a structure-driven approach based on a peptidic DTI structure in complex with bovine thrombin.[20] Dabigatran etexilate (**10**) is a double prodrug; after oral administration to humans the pharmacologically active component, dabigatran (**9**), is released very rapidly by esterase-mediated hydrolysis, with an absolute bioavailability of 6–7%.[21]

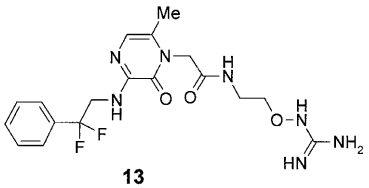
A further peptidic thrombin inhibitor from AstraZeneca, the prodrug AZD-0837[22] (**11**, atecegatran fexenetil) with an oral bioavailability of 22–52%,[23] was intended for Phase III clinical trials in 2008. Owing to inadequate stability of the tablet formulation, this study was postponed to 2009, and since then, no start of another study has been announced.

Other basic, but non-amidinic inhibitors have also been studied in the clinic: MCC-977 (**12**, sofigatran; Scheme 5)[24] from Mitsubishi Tanabe Pharma, with an aminocyclohexyl residue as S1 binder, entered Phase II studies in 2003, but the development was terminated in November 2007. Scientists from Johnson & Johnson recently reported inhibitors with an oxyguanidinyl P1 residue, which have a significantly reduced pK_a value of 7–7.5 compared to that of guanidine (pK_a 13–14):[25] thus RWJ-671818 (**13**) shows not only high efficacy in various in vivo thrombosis models but also high bioavailabil-



12

sofigatran (MCC-977, Mitsubishi)
FIIa: K_i = 9.6 nM



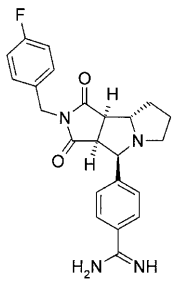
13

RWJ-671818 (J & J)
FIIa: K_i = 1.3 nM

Scheme 5. Thrombin inhibitors.

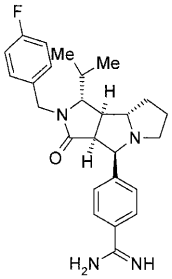
ity after oral dosage in dogs. Because of the advantageous preclinical profile, RJW-671818 was investigated in healthy subjects during Phase I studies, which were completed successfully.

Banner et al. were able to show that fluoro interactions are well suited for increasing the affinity and the selectivity for the substrate binding pocket of thrombin: The 4-fluoro substituent on the benzylic P4 residue in a series of tricyclic thrombin inhibitors such as **14** (Scheme 6) resulted in a five-



14

FIIa: K_i = 57 nM



15

FIIa: K_i = 5 nM

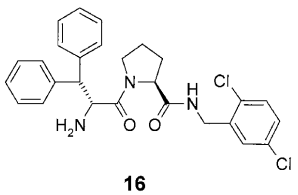
Scheme 6. Thrombin inhibitors with 4-fluoro substitution in the P4 side.

to tenfold increase in the affinity over that of corresponding ligands with other fluorination patterns. The analysis of the X-ray structure of the thrombin–ligand complex shows favorable C–F···H–C_α–C=O– and C–F···C=O interactions of the 4-fluoro substituent on the ligand with the H–C_α–C=O unit of Asn98. In addition the introduction of an isopropyl group to the basic tricyclic framework of ligand **15** resulted in improved filling of the S2 pocket and thus to an additional increase in affinity by a factor of 11.[26]

In order to circumvent the requirement for prodrug formation to achieve oral bioavailability for basic inhibitors, an intensive search for nonbasic P1 residues has been on since the beginning of the 1990s. In 1993, in cooperation with the London Thrombosis Research Institute and Cambridge University, Sandoz Pharma published the first example of a direct thrombin inhibitor with a neutral P1 residue, a peptidic boronic acid ester derivative with a methoxypropyl P1 side chain which binds covalently. The boronic acid ester group is hydrolyzed under physiological conditions to the boronic acid, which in turn forms an adduct with Ser195 in thrombin.[27]

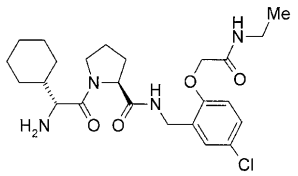
In 1998, Merck (USA) reported on noncovalent inhibitors with neutral P1 residues[28] in which a chloro substituent interacts with the amino acid Tyr228 in the S1 pocket. This new binding mode, first published by Merck (USA), which replaces the direct electrostatic interaction of a basic P1 residue with amino acid Asp189, is also used in many nonbasic factor Xa inhibitors. More recently, it has been speculated that additional halogen binding interactions with carbonyl groups of the peptide backbone might play an important role.[42]

Compound **16** (Scheme 7) with a dichlorobenzyl S1 residue is indeed orally bioavailable albeit very lipophilic; this property is associated with high plasma protein binding



16

FIIa: K_i = 3 nM



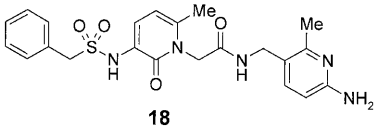
17

FIIa: K_i = 0.74 nM

Scheme 7. Thrombin inhibitors with nonbasic P1 groups.

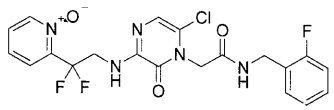
and inadequate efficacy in in vivo thrombosis models. Attempts to make this substructure class significantly more polar without loss of potency and bioavailability resulted in **17**, for example. With this class of compounds it remains, however, difficult to extend their half-life without losing the achieved advantageous profile with respect to potency, selectivity, in vivo efficiency, and oral bioavailability.

In parallel work, peptidomimetic basic structures with a central pyridinone building block have been investigated: the pyridinone derivative L-374,087 (**18**; Scheme 8), which has inadequate oral bioavailability and metabolic stability, was optimized over several learning cycles to the pyrazinone derivative **19**,[29] which has adequate bioavailability and improved metabolic stability and solubility. Investigations on the mode of binding showed[29] that the fluoro-substituted benzylamino residue is located in the S1 pocket and the



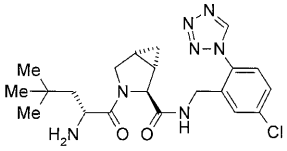
18

L-374,087
FIIa: K_i = 0.5 nM



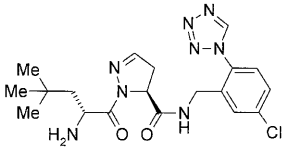
19

FIIa: K_i = 2.3 nM



20

FIIa: IC50 = 1 nM



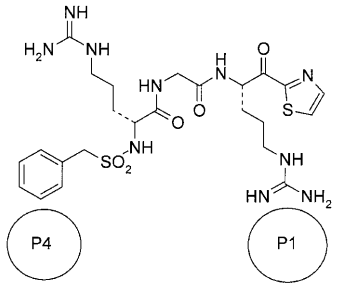
21

FIIa: IC50 = 3.5 nM

Scheme 8. Thrombin inhibitors with nonbasic P1 groups.

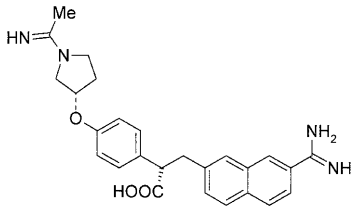
bioavailable.[32] This was the stimulus for an intensive search for small, orally bioavailable inhibitor molecules.[33]

Initially, inhibitor design concentrated on dibasic peptide analogues which take part in reversible covalent binding with the catalytic serine and simulate the transition state of the enzyme–substrate complex (Scheme 9).[34] In compound **22**



22

FXa: IC50 = 0.5 nM



23

DX-9065a
FXa: IC50 = 70 nM

Scheme 9. Dibasic FXa inhibitors.

chloropyridazinone residue in the S2 pocket. The *N*-oxide group, which not only contributes significantly to the improved solubility but also doubles the potency, is exposed to the solvent and does not appear to enter into a specific interaction with the thrombin backbone. Moreover, the π-electron-deficient P3 pyridyl-*N*-oxide appears to reinforce the edge-to-face interaction with the π-electron-rich amino acid Trp215 in the S4 pocket.

More recent patent applications from AstraZeneca claim potent azabicylohexanes such as **20**[30] and dihydro-1*H*-pyrazoles such as **21**[31] with chlorotetrazolylbenzyl residues, whereby the chloro substituent interacts with the amino acid Tyr228 in the S1 pocket, the annulated cyclopropyl ring is localized in the S2 pocket and the *tert*-butyl group in the S4 pocket.

Despite many and structurally heterogenous approaches to the discovery of direct thrombin inhibitors over the last 30 years, only one drug made it onto the market: the double prodrug dabigatran (**10**) from Boehringer Ingelheim.

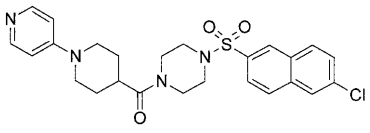
4. Factor Xa Inhibitors

The best-known natural direct FXa inhibitors are antistasin (119 amino acids) from the leech *Hementeria officinalis* and the tick anticoagulant peptide (TAP, 60 amino acids) from the tick *Ornithodorus moubata*; they are, however, not orally

from COR Therapeutics this binding is effected by the ketone group, while the thiazolyl residue forms a hydrogen bond with the histidine of the catalytic triad. The arginine-like residue interacts with the carboxylic acid of Asp189 in the S1 pocket. At the P4 end of the molecule the benzylsulfonyl residue is located in the hydrophobic pocket, while the second arginine side chain forms hydrogen bonds with the cation hole. However, this compound has three disadvantages: its peptidic nature results in unfavorable pharmacokinetic properties and short half-lives. Its basicity leads to poor oral bioavailability since large molecules bearing a positive charge under physiological conditions are poorly absorbed from the gastrointestinal tract. Its mode of action based on covalent modifications also may be accompanied by an increased risk for toxic side effects.

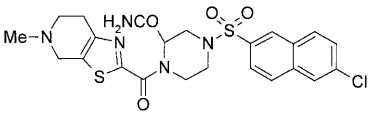
In 1993, Daiichi introduced the first noncovalent, nonpeptidic FXa inhibitor: DX-9065a (**23**).[35] This compound is also dibasic; its oral bioavailability is therefore poor. An X-ray structure analysis with FXa shows that the amidinylnaphthyl group interacts with Asp189 in the S1 pocket through a salt bridge and the pyrrolidine ring binds with the aryl-binding S4 domain through π–cation interactions. In addition, the protonated acetimidoyl group is located in the cation hole, where the positive charge interacts with Glu97 and Lys96.

There has been intensive research on less basic arginine mimetics in order to achieve a compromise between affinity for the enzyme and oral bioavailability.[36] In 1996, Zeneca published FXa inhibitors such as **24** (Scheme 10) with only



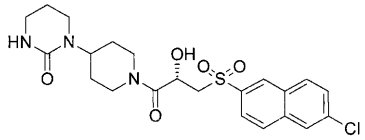
24

FXa: IC50 = 3 nM



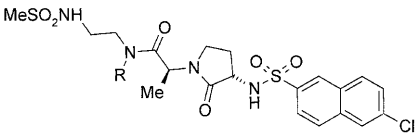
25

FXa: IC50 = 24 nM



26

TAK-442
FXa: IC50 = 3.5 nM



27

a (R = H): FXa: K_i = 39000 nM
b (R = *i*Pr): FXa: K_i = 1 nM

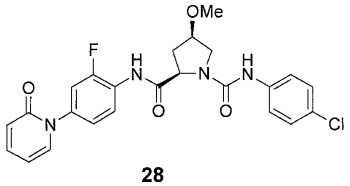
Scheme 10. FXa inhibitors with chloronaphthyl residues as the P1 group.

weakly basic groups.[37] For a long time the pyridylpiperidinyl group of **24** was erroneously assigned to the basic P1 group.[36] An X-ray structure analysis with trypsin showed, however, that the chloronaphthyl group is located in the S1 pocket. It was apparent that a comparable orientation is also true for FXa.[38] Further work by Daiichi resulted in compound **25**, for which this binding mode with chloronaphthalene in the S1 side could be demonstrated explicitly.[39]

Another chloronaphthyl compound, TAK-442 (**26**), is in Phase II clinical development for the treatment of pulmonary embolism, stroke, and acute coronary syndrome.[40] Here, too, the X-ray structure of the complex with human FXa confirmed that the 6-chloronaphthyl group occupies the S1 substrate binding pocket, whereby the chlorine atom has a hydrophobic interaction with the arene of Tyr228. The sulfonyl oxygen atom is involved in a hydrogen bond with Gln192. In addition, hydrogen bonds between the oxygen atoms of the amide carbonyl group and the hydroxy group and the backbone NH units of Gly219 and Gly216, respectively, can be observed. The cyclic urea interacts with the aromatic amino acids of the S4 pocket, whereby the carbonyl group forms a water-mediated contact with the amide oxygen atom of Lys96. In TAK-442 (**26**) the hydroxy group leads not only to a greater affinity for FXa, but through steric hindrance it also stabilizes the molecule toward hydrolytic cleavage of the neighboring amide. The hydroxy compounds were originally found when the metabolism of the nonhydroxylated compounds was investigated.

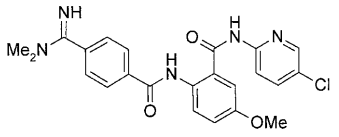
An impressive 40000 fold increase in potency was achieved starting with an inhibitor from Glaxo, **27a** (R = H), by the introduction of an isopropyl group into the P4 region (**27b**; R = *i*Pr).[41] This can be explained by the increased lipophilic interaction of the isopropyl group with the aromatic S4 pocket. Another reason is the displacement of solvate water from the lipophilic pocket, an effect not possible with the related weakly active NH compound.[42]

Eribaxaban (**28**),[43] a former development candidate from Pfizer, has a chloroanilino group instead of a chloronaphthyl residue at the P1 position (Scheme 11). Because of the



28

eribaxaban
FXa: IC50 = 0.32 nM



29

betrixaban
(PRT-54021, PRT-021)
FXa: IC50 = 1.5 nM

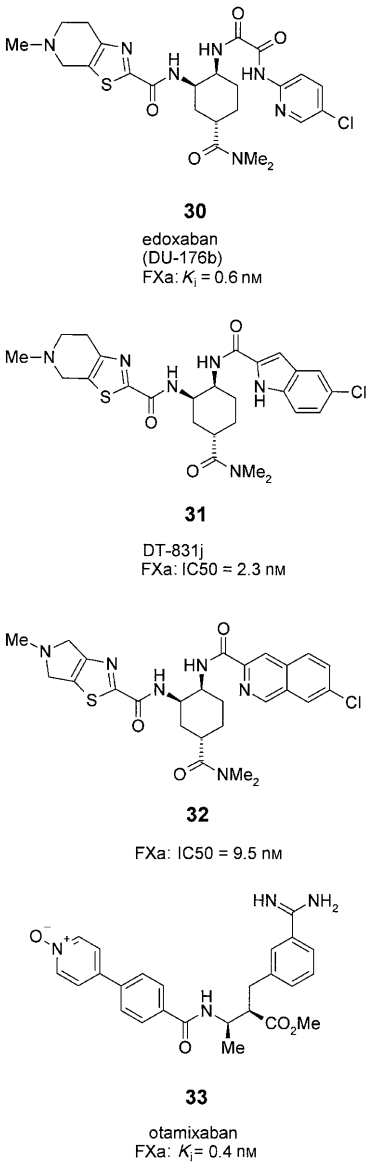
Scheme 11. FXa inhibitors with chloroanilino and chloropyridyl residues as the P1 group.

carcinogenic properties of chloroaniline it was replaced by chloropyridine. This group is a component of betrixaban (**29**),[62] a development candidate from Portola/Merck (US). It was shown with analogous derivatives that in the S1 pocket the chloropyridyl unit not only forms the Cl–π interaction with Tyr228, but also a hydrogen bond to Gly218 through the amide NH group.[61] Although betrixaban (**29**) comprises a benzamidinyl group protonated under physiological conditions which forms the π–cation interactions in the lipophilic S4 pocket, it shows surprisingly good oral bioavailability of almost 60% in monkeys. Since a corresponding derivative with a cyclic guanidine group at this position with a pK_a value of 14 has only very low oral bioavailability (< 1% in rats), the reason evidently lies in the lower pK_a value of 10 of the benzamidine. Favorably effective in this context is also *N*,*N*-dimethyl substitution, which is not tolerated sterically in the S1 pocket but fits into the S4 pocket.[44]

An important aspect of drug safety concerns the inhibition of the cardiac hERG potassium channels which is repeatedly observed as an adverse effect with active compounds and which increases the risk of cardiac arrhythmias. Lipophilic molecules with positively charged amines and benzamidines, as in betrixaban, are particularly affected by this problem since these mimic the positive charge of the potassium cation.[45] The work with betrixaban revealed that chloroanthranilamide groups in the central part of the inhibitor molecule cause significant hERG inhibition. The replacement of the chlorine atom by a less lipophilic methoxy group led to a 16-fold decrease in hERG inhibition, which was sufficient for starting clinical studies;[62] many other derivatives required intramolecular compensation of the positive charge by a carboxylate group.[45]

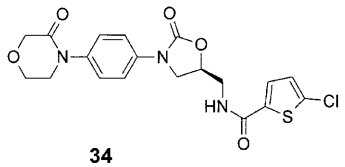
Extensive work has been carried out by Daiichi and was the subject of several publications and patent applications containing more than one-thousand pages.[46] The result of this work was edoxaban (**30**; Scheme 12),[47] for which the application for marketing approval was filed in Japan in 2010.[48] A dimethylamide unit was attached in order to improve the metabolic stability of the cyclohexanediamine center. A chloropyridyl residue represents the P1 group. A backup candidate, DT-831j (**31**), was prepared starting from **30** by including the carbonyl group for annulation to give a bicyclic chloroindole.[49] This resulted in an additional hydrogen bond of the indole NH unit with the Gly218 oxygen group in the S1 pocket. The X-ray crystal structure with the related compound **32** (with a chloroisoquinolinyl P1 residue) showed a water-mediated hydrogen bond between the ring nitrogen atom and Ser195 and a hydrogen bond between the isoquinolinyl H4 atom and the Gly218 carbonylamide unit of the enzyme. In **31** the six-membered ring annulated P4 residue tetrahydro-5-methylthiazolo[5,4-c]pyridine was replaced with a dihydropyrrolo[3,4-d]thiazolyl residue. This led to reduced lipophilicity, lower plasma protein binding, higher anticoagulant activity in plasma, and improved solubility making its oral activity less susceptible to food intake.[49c] Otamixaban (**33**)[50] from Aventis is currently in Phase III of clinical development for use in acute coronary syndrome. It contains a benzamidinyl residue as the P1 group and the pyridyl-*N*-oxide residue occupies the S4 pocket forming a water-mediated hydrogen bond to the carbonyl oxygen atom of Ile175. With a short half-life it is suitable only for intravenous administration.

Rivaroxaban (**34**; Scheme 13) from Bayer came onto the market in 2008 as the first direct, orally active factor Xa inhibitor.[51] Here a chlorothienyl unit assumes the function of the P1 group, whereby the chlorine atom participates in a hydrophobic edge-to-face Cl···π interaction with the arene of Tyr228 and displaces a water molecule positioned at this site, which provides a further entropic binding contribution. The morpholinone carbonyl group of the P4 residue leads to a vertical twisting of the morpholinone ring relative to the arene and polarizes the ring CH_2 group above Trp215, which reinforces the CH_2···π interaction.[42] The pattern of P1 and P4 residues present in rivaroxaban proved to be particularly efficient and was also adopted in other structures such as **35**[52] and **50**.



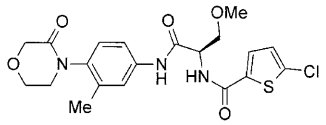
Scheme 12. FXa inhibitors.

Razaxaban (**37**; Scheme 14) was in Phase II of clinical development.[53] The molecule was derived from the FXa inhibitor DPC423 (**36**), which is less selective for trypsin. As the benzylamine P1 group in DPC423 is less basic than benzamidine, DPC423 was orally bioavailable. Razaxaban has instead a nonbasic and larger P1 aminobenzisoxazolyl residue, whereby the amino group is responsible for the interaction with Asp189 in the S1 pocket and with Gly218. Due to the smaller Ala190, the S1 pocket of factor Xa is larger than that of trypsin which has Ser190 at the same site. Thus it was possible to increase the relative affinity for FXa compared to that for trypsin from 440-fold to $>25\,000$-fold. However, the new heterocycle together with the (methylsulfonyl)methyl unit of DPC423 worsened the solubility of the molecule to the extent that an imidazolyl residue with a (dimethylamino)methyl unit had to be incorporated on the P4 side. This led to an improvement in solubility and intestinal permeability and to a reduction in plasma protein binding.



34

rivaroxaban (Xarelto(R))
FXa: IC50 = 0.7 nM



35

EMD 495235
FXa: IC50 = 5.5 nM

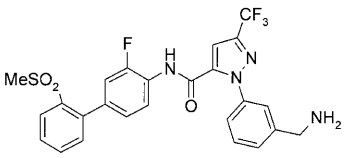
Scheme 13. FXa inhibitors with chlorothienyl residues as the P1 group and a morpholinonyl unit as the P4 group.

The N3 nitrogen atom of the imidazole ring forms a hydrogen bond to Glu97 of the S4 pocket.

Metabolism and toxicology of a development candidate must also be considered. The inhibition and induction of cytochrome P450 in the liver lead to adverse drug reactions. With DPC423 the reactive intermediates formed by CYP enzymes during the course of the metabolism of the benzylamino group can give rise to toxic effects.[54] In addition with members of the DPC423 class of substances the amidic linkage of the P4 part was metabolically unstable and led to the release of mutagenic methylsulfonylbiphenylaniline.[55] During further optimization to the clinical candidate apixaban (**38**) the aniline unit was connected to the pyrazole ring by an additional bridge in order to stabilize the linkage.

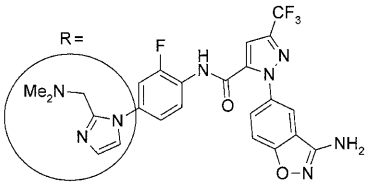
Apixaban (**38**) from Pfizer/BMS is another nonbasic FXa inhibitor for which marketing approval has been filed. The X-ray structure analysis of this 0.08 nM inhibitor shows that in the S1 pocket the *p*-methoxy group displaces an unfavorably bound water molecule. The pyrazole N2 nitrogen atom interacts with the backbone of Gln192 and the carbonyl oxygen atom of the carboxamide interacts with the NH unit of Gly216. The orientation of the lactam phenyl residue in the S4 pocket indicates an edge-to-face interaction with Trp215.[55]

The development of new factor Xa inhibitors continues unabated: recently, a new P1 group was introduced in the form of the triazolinone phenyl residue,[56] which is actually too large for the S1 pocket. The crystal structure of FXa with the inhibitor **39**, derived from **38**, shows, however, that in this case the Asp189 residue is twisted out of its normal position and thus a larger pocket is provided to accommodate the triazolinone phenyl residue. The carbonyl group of the triazolinone unit forms a hydrogen bond to the Asp189 carboxylate group mediated through a water molecule. A further hydrogen bond to the other carboxylate oxygen atom is also mediated by a water molecule and originates from a triazolinone N atom. The other triazolinone NH unit forms a hydrogen bond to Gly218. The compound is selective because in the S1 pocket of thrombin the Asp189 side chain "defends" itself from being forced back through an electrostatic repulsion to Asp221; factor Xa contains merely an uncharged Ala221 at this position.



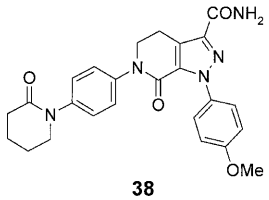
36

DPC423
FXa: K_i = 0.15 nM



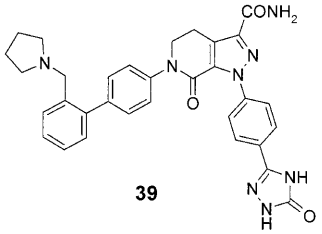
37

razaxaban
FXa: K_i = 0.19 nM
R = 2-PhCH$_2$NH$_2$ FXa: K_i = 2.0 nM
R = 2-PhCH$_2$NMe$_2$ FXa: K_i = 0.2 nM
R = 2-PhCH$_2$N$^+$Me$_3$ Br$^-$ FXa: K_i = 0.05 nM



38

apixaban
FXa: K_i = 0.08 nM



39

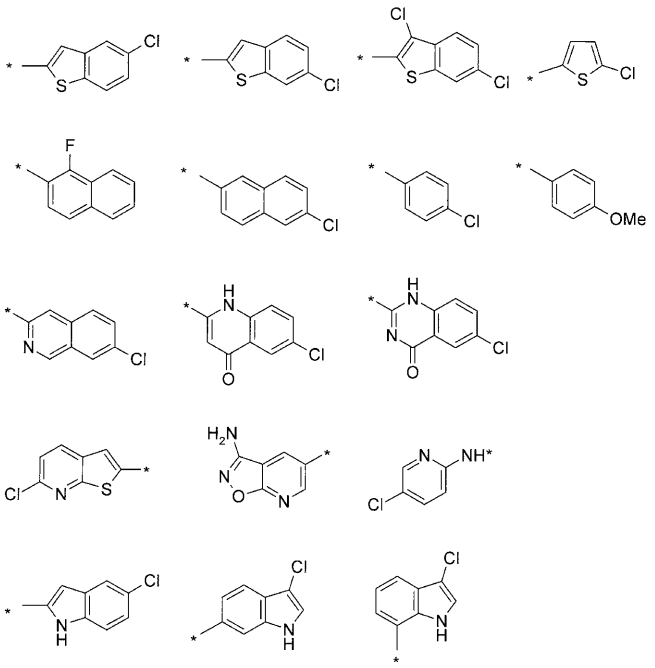
FXa: K_i = 0.25 nM
PT EC2x = 4.4 μM

Scheme 14. FXa inhibitors of the apixaban type.

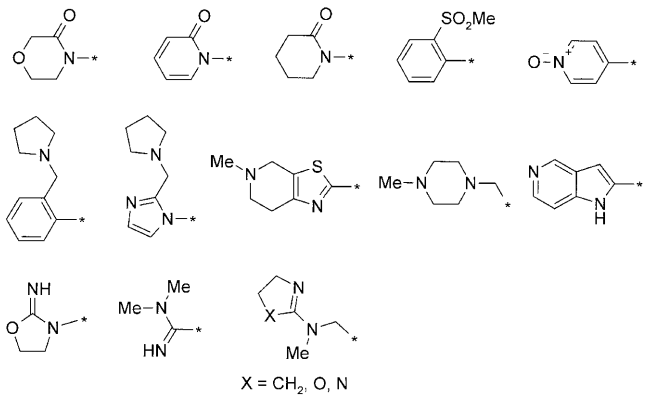
Many FXa inhibitors are composed of three pharmacophores: the P1 residue, the P4 residue, and a central part. Frequently used pharmacophores are listed here.

Preferred nonbasic P1 building blocks include chlorothiophene, chlorobenzothiophene, chlorobenzene, chloronaphthalene, 4-methoxybenzene, chloroisoquinoline,[57] and chloroindole (Scheme 15).[49]

The lipophilic S4 pocket is preferably addressed by substituted heterocycles which can also accommodate a positive charge in order to interact with the "cation hole" (Scheme 16). Examples are methylsulfonylbenzene (DPC423), pyridine-*N*-oxide (otamixaban), pyridine, pyrrolopyridine,[58] tetrahydro-5-methylthiazolo[5,4-c]pyridine (edoxaban), pyrazole, and imidazole as well as nonaromatic



Scheme 15. Preferred nonbasic or weakly basic P1 building blocks in FXa inhibitors.

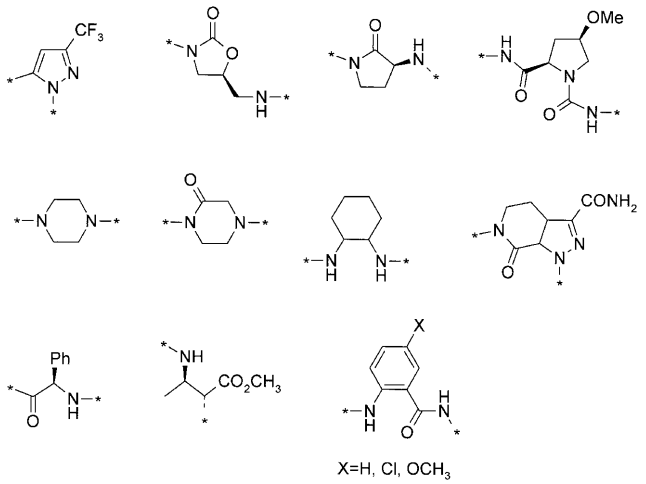


Scheme 16. Preferred P4 building blocks in FXa inhibitors.

amides, lactams (apixaban), morpholinones (rivaroxaban), pyridones (eribaxaban), cyclic amines,[59] methylpiperazine,[60] methylaminooxazolines and -imidazoles,[61] oxazolidinimines, amidines (betrixaban),[62] and guanidines.

It has been possible to show that stepwise methylation of a terminal P4 aminoalkyl group leads to an increase in the cation–π interaction to the amino acid side chains in the aromatic pocket, whereby the tertiary and the quaternary ammonium also profit from decreasing desolvation energies.[63] This also explains why in a series of razaxaban derivates **37** (Scheme 14) the K_i value for FXa decreases in the series 2-PhCH_2NH_2 (K_i=2.0 nM) > 2-PhCH_2NMe_2 (K_i=0.2 nM) > 2-Ph-CH_2-$N^+Me_3Br^-$ (K_i=0.05 nM), but absorption through the intestinal wall deteriorates.[64]

For the central part (Scheme 17), to which the P1 and P4 residues are bonded, building blocks such as oxazolidinones, anthranilamides, cyclic diamines, indazoles, pyrazoles, piperazinones, and indoles have been described. The bonding to the P1 and P4 residues occurs through methylene, amide, or sulfonamide bonds, whereby a phenylene, chlorothienyl, or piperidinyl bridge to the P4 residue is frequently interposed.



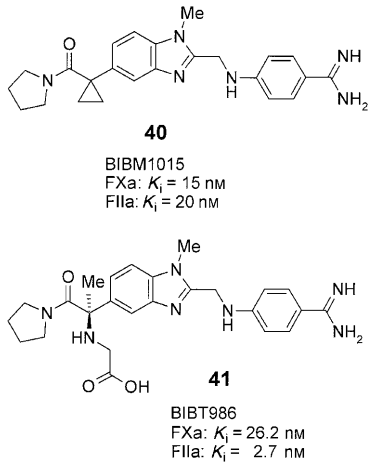
Scheme 17. Preferred central building blocks in FXa inhibitors.

5. Dual Thrombin/Factor Xa Inhibitors

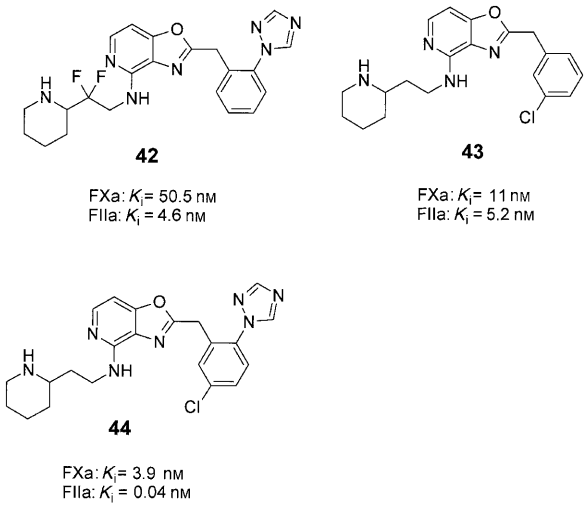
If a thrombin inhibitor and a factor Xa inhibitor are combined at an antithrombotic dose that is for each below the threshold for an antithrombotic effect, thrombosis models show that concurrent direct inhibition of both targets not only produces an additive but even a synergistic antithrombic effect.[65,66] These findings offer hope that a dual therapy could have a larger therapeutic window. While still displaying the desired antithrombic effect owing to the synergism, the lower dosage could lead to fewer adverse effects such as hemorrhage. For this reason several groups have also started to search for dual inhibitors.[67]

In 2001, Boehringer Ingelheim reported that the 1-methylbenzimidazole unit in a series of their inhibitors is able to fill both the small solvent-accessible pocket in factor Xa and the larger hydrophobic S2 pocket in thrombin.[68] One of the most potent dual inhibitors here is BIBM1015 (**40**; Scheme 18). Another derivative from this series, BIBT986 (**41**, tanogitran),[69] was taken into clinical development and successfully investigated in humans in a model of endotoxin-induced coagulation;[70] however, since then no further development of this compound has been reported.

In 2004, Merck (US) reported on a new series of oxazolopyridines (**42**–**44**; Scheme 19) whose optimization during the course of the project also led to dual inhibitors.[71] In this case the triazolyl- and/or chloro-substituted arene interacts with the S1 pocket, the oxazolopyridine with the S2 pocket, and the piperidyl group with the S3 pocket. The exocyclic NH group and the nitrogen atom of the oxazolo ring both form a hydrogen bond with Gly216, while the nitrogen atom of the P3 piperidine unit forms an intramolecular hydrogen bond with the aminopyridine ring nitrogen atom.



Scheme 18. Dual thrombin/FXa inhibitors from Boehringer Ingelheim.

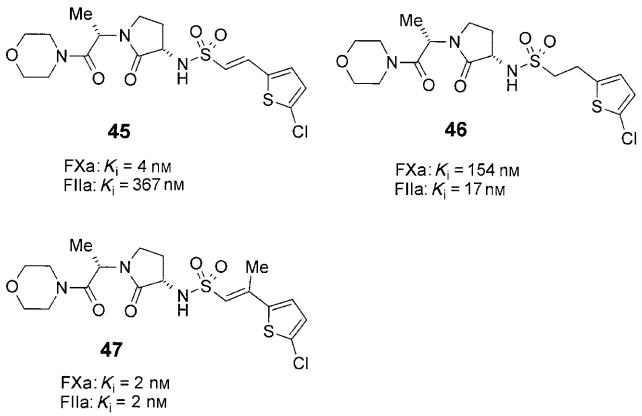


Scheme 19. Dual thrombin/FXa inhibitors from Merck (US).

Compound **44** was the most potent inhibitor but showed a very low oral bioavailability of $< 1\,\%$.

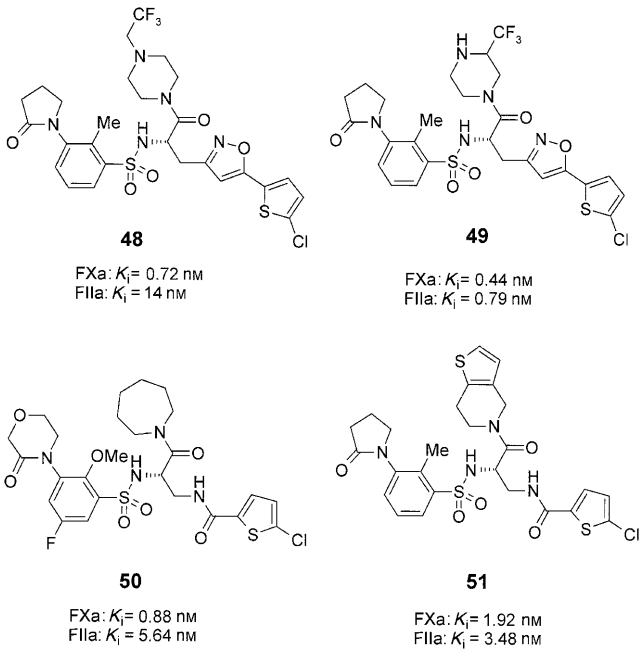
Small variations in the P1 part of the factor Xa inhibitor **45** from GlaxoSmithKline led to the saturated **46**, displaying stronger binding activity to thrombin than to factor Xa. Compound **47** with an additional methyl group attached to the double bond was a surprisingly potent dual inhibitor (Scheme 20).[72] All three compounds bind to thrombin in a similar mode: The chlorothienyl group functions as the S1 binder, the carbonyl group of pyrrolidine interacts with the protein backbone, and the morpholine ring occupies the S2 pocket; the S3 and S4 pockets remain unoccupied. In the case of binding to factor Xa, the chlorothienyl group and the carbonyl group of the pyrrolidine participate in analogous interactions; however, in contrast to thrombin, the alanylamide group of the morpholine unit of all three compounds is localized in the S4 pocket. The differences in the inhibitor profiles of the three compounds are attributable to different conformations of the respective sulfonamide.

Further very potent dual thrombin/factor Xa inhibitors such as **48**–**51** from Sanofi-Aventis were published in 2009



Scheme 20. Dual thrombin/FXa inhibitors from GlaxoSmithKline.

(Scheme 21);[73,74] however, no information is available on the further development status of this class of compounds.



Scheme 21. Dual thrombin/FXa inhibitors from Sanofi-Aventis.

6. Comparative Observations on the Development of Compounds as FIIa and FXa Inhibitors

Why did the pharmaceutical industry succeed faster with FXa inhibitors than with thrombin inhibitors in spite of the later entry into drug-discovery efforts?

To answer this question it is useful to analyze the binding pockets of these two closely related enzymes in detail. When the sequences of the protease domains of important serine proteases involved in blood clotting (Figure 1) are compared, it can be recognized that thrombin and FXa share a sequence identity of 37 %, but in the region of amino acid position 60 thrombin has a larger loop than all other proteases. In the substrate binding pocket this leads to a larger, mainly

lipophilic S2 pocket. The S1 pocket of thrombin is highly similar to that of FXa. The amino acids at position 227 (thrombin: Phe227, FXa: Ile227) are different. But since they extend inwards into the protein, this has little effect on the shape and properties of the S1 surface. In addition thrombin-Glu192 is exchanged for FXa-Gln192 at the edge of the S1 pocket. Amino acids at this position are usually very flexible and therefore rarely involved in inhibitor–protein interactions. Significant differences arise, however, in the region of the S4 pocket: in FXa this is lined by two aromatic amino acids (Tyr99, Phe174), whereas thrombin contains an Ile and a Leu there. The bottom of the S4 pocket is formed by a Trp215 in both proteins. Further differences may also be seen in the S3 pocket: in both proteases it forms merely a flat indentation on the protein surface. With thrombin there are possibilities for interactions of polar substituents with Thr172 in the S3 pocket, whereas with FXa the corresponding Ser172 is shielded by the larger Phe174.

Apart from the deep S1 pocket, thrombin has a rather flat, lipophilic substrate binding site. In contrast, FXa with the two aromatic amino acids in the S4 pocket forms a deeper inhibitor binding site. In the case of FXa the replacement of polar P1 groups by lipophilic substituents (e.g. a chlorothienyl group) brought about the breakthrough to inhibitors with very good pharmacokinetic properties. By means of a flat, π-electron rich P4 group very potent (subnanomolar) inhibitors with balanced lipophilicity can be obtained. The high affinity of thrombin inhibitors that made it to the market results from ionic interactions with the S1 and the S3 pockets and lipophilic contacts with S2 and S4 pockets. These compounds are charged and have a relatively high molecular weight, which is reflected in moderate pharmacokinetics. Since the S1 pockets of thrombin and FXa are similar, the chlorothienyl residue can be used as the P1 group in both FXa and thrombin inhibitors. Due to the lack of aromatic amino acids and its more shallow surface, the S4 pocket of thrombin provides only a limited energetic contribution to the interaction with inhibitors which is in contrast to FXa. With thrombin, additional binding energy may be obtained only in the S2 pocket using relatively lipophilic P2 groups. However, thus far it has not been possible to optimize such highly lipophilic compounds for use in humans.

In summary: Although FXa and Thrombin are rather similar, even small differences in their substrate binding pockets can tip the scales for success in inhibitor optimization. In this context it is far more difficult to find thrombin inhibitors with both high binding affinity and beneficial pharmacokinetic properties.

Received: July 26, 2010

Translated by Dr. David Le Count, Congleton (UK)

[1] J. A. López, J. Chen, *Thromb. Res.* **2009**, *123 Suppl. 4*, S30 – S34.
[2] Y. Nakamura, K. Nakamura, K. Fukushima-Kusano, K. Ohta, H. Matsubara, T. Hamuro, C. Yutani, T. Ohe, *Thromb. Res.* **2003**, *111*, 137 – 142.
[3] M. Petitou, C. A. A. van Boeckel, *Angew. Chem.* **2004**, *116*, 3180 – 3196; *Angew. Chem. Int. Ed.* **2004**, *43*, 3118 – 3133.
[4] D. Kubitza, S. Haas, *Expert Opin. Invest. Drugs* **2006**, *15*, 843 – 855.
[5] J. Dodt, H.-P. Müller, U. Seemüller, J.-Y. Chang, *FEBS Lett.* **1984**, *165*, 180 – 184.
[6] F. Markwardt, *Naturwissenschaften* **1955**, *42*, 537 – 538.
[7] P. Walsmann, F. Markwardt, *Pharmazie* **1981**, *36*, 653 – 660.
[8] J. M. Maraganore, P. Bourdon, J. Jablonski, K. L. Ramachandran, J. W. Fenton, *Biochemistry* **1990**, *29*, 7095 – 7101.
[9] J. Stürzebecher, F. Markwardt, B. Voigt, G. Wagner, P. Walsmann, *Thromb. Res.* **1983**, *29*, 635 – 642.
[10] R. Kikumoto, Y. Tamao, T. Tezuka, S. Tonomura, H. Hara, K. Ninomiya, A. Hijikata, S. Okamoto, *Biochemistry* **1984**, *23*, 85 – 90.
[11] S. Sherry, N. Alkjaersig, A. P. Fletcher, *Am. J. Physiol.* **1965**, *209*, 577 – 583.
[12] a) D. Banner, P. Hadvary, *J. Biol. Chem.* **1991**, *266*, 20085 – 20093; b) H. Brandstetter, D. Turk, H. W. Hoeffken, D. Grosse, J. Stürzebecher, P. D. Martin, B. F. P. Edwards, W. Bode, *J. Mol. Biol.* **1992**, *226*, 1085 – 1099.
[13] a) W. Bode, R. Huber, T. J. Rydel, A. Tulinsky in *Thrombin Structure and Function* (Ed.: L. J. Berliner), Plenum, New York, **1992**, p. 3 – 61; b) D. W. Banner, P. Hadvary, *Adv. Exp. Med. Biol.* **1993**, *340*, 27 – 33.
[14] a) C. Kettner, E. Shaw, *Thromb. Res.* **1979**, *14*, 969 – 973; b) B. Walker, P. Wikstom, E. Shaw, *Biochem. J.* **1985**, *230*, 645 – 650.
[15] W. Bode, I. Mayr, U. Baumann, R. Huber, S. R. Stone, J. Hofsteenge, *EMBO J.* **1989**, *8*, 3467 – 3475.
[16] S. Bajusz, E. Széll, E. Barabás, D. Bagdy, Z. Mohai (Richter, Gedeon, Vegyeszeti Gyar Rt., Hungary), US 4,346,078, **1982**.
[17] U. G. Eriksson, U. Bredberg, K.-J. Hoffmann, A. Thuresson, M. Gabrielsson, H. Ericsson, M. Ahnoff, K. Gislen, G. Fager, D. Gustafsson, *Drug Metab. Dispos.* **2003**, *31*, 294 – 305.
[18] D. Gustafsson, J.-E. Nyström, S. Carlsson, U. Bredberg, U. Eriksson, E. Gyzander, M. Elg, T. Antonsson, K.-J. Hoffmann, A.-L. Ungell, H. Sörensen, S. Någård, A. Abrahamsson, R. Bylund, *Thromb. Res.* **2001**, *101*, 171 – 181.
[19] L. A. Sorbera, J. Bozzo, J. Castaner, *Drugs Future* **2005**, *30*, 877 – 885.
[20] N. H. Hauel, H. Nar, H. Priepke, U. J. Ries, J.-M. Stassen, W. Wienen, *J. Med. Chem.* **2002**, *45*, 1757 – 1766.
[21] S. Blech, T. Ebner, E. Ludwig-Schwellinger, J. Stangier, W. Roth, *Drug Metab. Dispos.* **2008**, *36*, 386 – 399.
[22] S. B. Olsson, L. H. Rasmussen, A. Tveit, E. Jensen, P. Wessman, S. Panfilov, K. Wåhlander, *Thromb. Haemostasis* **2010**, *103*, 604 – 612.
[23] S. J. Johansson, M. Cullberg, A. Ekdahl, K. Wåhlander, K. Duner, U. G. Eriksson, *Basic Clin. Pharmacol. Toxicol.* **2009**, *105 (Suppl. 1)*, ABST P78.
[24] a) M. Nakajima, M. Suga, K. Sugawara, K. Katsu, S. Yuki, J. Anabuki, T. Yamamoto, Y. Abe, Y. Kitada, *J. Thromb. Haemostasis* **2007**, *5 Supplement 2:* P-T-643; b) M. Nakajima, M. Suga, K. Sugawara, K. Yamada, J. Anabuki, T. Yamamoto, Y. Kitada, *J. Thromb. Haemostasis* **2007**, *5 Supplement 2*: P-T-644.
[25] T. Lu, T. Markotan, S. K. Ballentine, E. C. Giardino, J. Spurlino, K. Brown, B. E. Maryanoff, B. E. Tomczuk, B. P. Damiano, U. Shukla, D. End, P. Andrade-Gordon, R. F. Bone, M. R. Player, *J. Med. Chem.* **2010**, *53*, 1843 – 1856.
[26] J. A. Olsen, D. W. Banner, P. Seiler, B. Wagner, T. Tschopp, U. Obst-Sander, M. Kansy, K. Müller, F. Diederich, *ChemBioChem* **2004**, *5*, 666 – 675.
[27] a) C. Tapparelli, R. Metternich, C. Ehrhardt, M. Zurini, G. Claeson, M. F. Scully, S. R. Stone, *J. Biol. Chem.* **1993**, *268*, 4734 – 4741; b) G. Claeson, M. Philipp, E. Agner, M. F. Scully, R. Metternich, V. V. Kakkar, T. DeSoyza, L.-H. Niu, *Biochem. J.* **1993**, *296*, 309 – 312.
[28] a) W. C. Lumma, Jr., K. M. Witherup, T. J. Tucker, S. F. Brady, J. T. Sisko, A. M. Naylor-Olsen, S. D. Lewis, B. J. Lucas, J. P.

Vacca, *J. Med. Chem.* **1998**, *41*, 1011 – 1013; b) T. J. Tucker, S. F. Brady, W. C. Lumma, S. D. Lewis, S. J. Gardell, A. M. Naylor-Olsen, Y. Yan, J. T. Sisko, K. J. Stauffer, B. J. Lucas, J. J. Lynch, J. J. Cook, M. T. Stranieri, M. A. Holahan, E. A. Lyle, E. P. Baskin, I. W. Chen, K. B. Dancheck, J. A. Krüger, C. M. Cooper, J. P. Vacca, *J. Med. Chem.* **1998**, *41*, 3210 – 3219.

[29] C. S. Burgey, K. A. Robinson, T. A. Lyle, P. G. Natermet, H. G. Selnick, R. C. Isaacs, S. D. Lewis, B. J. Lucas, J. A. Krüger, R. Singh, C. Miller-Stein, R. B. White, B. Wong, E. A. Lyle, M. T. Stranieri, J. J. Cook, D. R. McMasters, J. M. Pellicore, S. Pal, A. A. Wallace, F. C. Clayton, D. Bohn, D. C. Welsh, J. J. Lynch, Jr., Y. Yan, Z. Chen, L. Kuo, S. J. Gardell, J. A. Shafer, J. P. Vacca, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1353 – 1357.

[30] J. Brånalt, D. Gustafsson, I. Nilsson, M. Polla, (AstraZeneca AB), WO 2009/004383, **2009**.

[31] J. Brånalt, D. Gustafsson, I. Nilsson, M. Polla, (AstraZeneca AB), WO 2009/157860, **2009**.

[32] B. Kaiser, *Drugs Future* **1998**, *23*, 423 – 436.

[33] Current reviews: a) A. Straub, S. Roehrig, A. Hillisch, *Curr. Top. Med. Chem.* **2010**, *10*, 257 – 269; b) D. J. P. Pinto, J. M. Smallheer, D. L. Cheney, R. M. Knabb, R. R. Wexler, *J. Med. Chem.* **2010**, *53*, 6243 – 6274.

[34] B.-J. Zhu, R. M. Scarborough, *Curr. Opin. Cardiovasc. Pulm. Renal Invest. Drugs* **1999**, *1*, 63 – 87, cit. Lit.

[35] a) T. Nagahara, N. Kanaya, K. Inamura, Y. Yokoyama, EP 540051, **1993**; b) T. Nagahara, Y. Yokoyama, K. Inamura, S.-I. Katakura, S. Komoriya, H. Yamaguchi, T. Hara, M. Iwamoto, *J. Med. Chem.* **1994**, *37*, 1200 – 1207.

[36] L. Peterlin Masic, *Curr. Med. Chem.* **2006**, *13*, 3627 – 3648.

[37] A. W. Faull, C. M. Mayo, J. Preston, A. Stocker (Zeneca Limited, Great Britain), WO 96/10022, **1996**.

[38] M. T. Stubbs, S. Reyda, F. Dullweber, M. Möller, G. Klebe, D. Dorsch, D. W. W. K. R. Mederski, H. Wurziger, *ChemBioChem* **2002**, *3*, 246 – 249.

[39] N. Haginoya, S. Kobayashi, S. Komoriya, T. Yoshino, M. Suzuki, T. Shimada, K. Watanabe, Y. Hirokawa, T. Furugori, T. Nagahara, *J. Med. Chem.* **2004**, *47*, 5167 – 5182.

[40] T. Fujimoto, Y. Imaeda, N. Konishi, K. Hiroe, M. Kawamura, G. P. Textor, K. Aertgeerts, K. Kubo, *J. Med. Chem.* **2010**, *53*, 3517 – 3531.

[41] R. J. Young, M. Campbell, A. D. Borthwick, D. Brown, C. L. Burns-Kurtis, C. Chan, M. A. Convery, M. C. Crowe, S. Dayal, H. Diallo, H. A. Kelly, N. P. King, S. Kleanthous, A. M. Mason, J. E. Mordaunt, C. Patel, A. J. Pateman, S. Senger, G. P. Shah, P. W. Smith, N. S. Watson, H. E. Weston, P. Zhou, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5953 – 5957.

[42] C. Bissantz, B. Kuhn, M. Stahl, *J. Med. Chem.* **2010**, *53*, 5061 – 5084.

[43] J. T. Kohrt, C. F. Bigge, J. W. Bryant, A. Casimiro-Garcia, L. Chi, W. L. Cody, T. Dahring, D. A. Dudley, K. J. Filipski, S. Haarer, R. Heemstra, N. Janiczek, L. Narasimhan, T. McClanahan, J. T. Peterson, V. I. Sahasrabudhe, R. Schaum, C. A. Van Huis, K. M. Welch, E. Zhang, R. J. Leadley, J. J. Edmunds, *Chem. Biol. Drug Des.* **2007**, *70*, 100 – 112.

[44] P. Zhang, L. Bao, J. Fan, Z. J. Jia, U. Sinha, P. W. Wong, G. Park, A. Hutchaleelaha, R. M. Scarborough, B.-Y. Zhu, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2186 – 2189.

[45] B.-Y. Zhu, Z. J. Jia, P. Zhang, T. Su, W. Huang, E. Goldman, D. Tumas, V. Kadambi, P. Eddy, U. Sinha, R. M. Scarborough, Y. Song, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5507 – 5512.

[46] T. Ohta, S. Komoriya, T. Yoshino, K. Uoto, Y. Nakamoto, H. Naito, A. Mochizuki, T. Nagata, H. Kanno, N. Haginoya, K. Yoshikawa, M. Nagamochi, S. Kobayashi, M. Ono, (Daiichi Sankyo Co Ltd.), WO 2003/016302, **2003**; WO 2004/058715, **2004**; WO 2003/000680, **2003**.

[47] T. Furugohri, K. Isobe, Y. Honda, C. Kamisato-Matsumoto, N. Sugiyama, T. Nagahara, Y. Morishima, T. Shibano, *J. Thromb. Haemostasis* **2008**, *6*, 1542 – 1549.

[48] Daiichi Files For Approval Of Blood Clot Drug, The Wall Street Journal (Online and Print), April 6, 2010.

[49] a) T. Nagata, T. Yoshino, N. Haginoya, K. Yoshikawa, M. Nagamochi, S. Kobayashi, S. Komoriya, A. Yokomizo, R. Muto, M. Yamaguchi, K. Osanai, M. Suzuki, H. Kanno, *Bioorg. Med. Chem.* **2009**, *17*, 1193 – 1206; b) K. Yoshikawa, A. Yokomizo, H. Naito, N. Haginoya, S. Kobayashi, T. Yoshino, T. Nagata, A. Mochizuki, K. Osanai, K. Watanabe, H. Kanno, T. Ohta, *Bioorg. Med. Chem.* **2009**, *17*, 8206 – 8220; c) K. Yoshikawa, S. Kobayashi, Y. Nakamoto, N. Haginoya, S. Komoriya, T. Yoshino, T. Nagata, A. Mochizuki, K. Watanabe, M. Suzuki, H. Kanno, T. Ohta, *Bioorg. Med. Chem.* **2009**, *17*, 8221 – 8233.

[50] K. R. Guertin, Y.-M. Choi, *Curr. Med. Chem.* **2007**, *14*, 2471 – 2481.

[51] S. Roehrig, A. Straub, J. Pohlmann, T. Lampe, S. Pernerstorfer, K.-H. Schlemmer, P. Reinemer, E. Perzborn, *J. Med. Chem.* **2005**, *48*, 5900 – 5908.

[52] W. W. K. R. Mederski, B. Cezanne, C. van Amsterdam, K.-U. Bühring, D. Dorsch, J. Gleitz, J. März, C. Tsaklakidis, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5817 – 5822.

[53] M. L. Quan, P. Y. S. Lam, Q. Han, D. J. P. Pinto, M. Y. He, E. Renhua, D. Christopher, C. G. Clark, C. A. Teleha, J.-H. Sun, R. S. Alexander, S. Bai, J. M. Luettgen, R. M. Knabb, P. C. Wong, R. R. Wexler, *J. Med. Chem.* **2005**, *48*, 1729 – 1744.

[54] a) A. E. Mutlib, J. Shockcor, S.-Y. Chen, R. J. Espina, D. J. Pinto, M. J. Orwat, S. R. Prakash, L.-S. Gan, *Chem. Res. Toxicol.* **2002**, *15*, 48 – 62; b) A. E. Mutlib, S.-Y. Chen, R. J. Espina, J. Shockcor, S. R. Prakash, L.-S. Gan, *Chem. Res. Toxicol.* **2002**, *15*, 63 – 75.

[55] D. J. P. Pinto, M. J. Orwat, S. Koch, K. A. Rossi, R. S. Alexander, A. Smallwood, P. C. Wong, A. R. Rendina, J. M. Luettgen, R. M. Knabb, K. He, B. Xin, R. R. Wexler, P. Y. S. Lam, *J. Med. Chem.* **2007**, *50*, 5339 – 5356.

[56] M. L. Quan, D. J. P. Pinto, K. A. Rossi, S. Sheriff, R. S. Alexander, E. Amparo, K. Kish, R. M. Knabb, J. M. Luettgen, P. Morin, A. Smallwood, F. J. Woerner, R. R. Wexler, *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1373 – 1377.

[57] K. Yoshikawa, S. Kobayashi, Y. Nakamoto, N. Haginoya, S. Komoriya, T. Yoshino, T. Nagata, A. Mochizuki, K. Watanabe, M. Suzuki, H. Kanno, T. Ohta, *Bioorg. Med. Chem.* **2009**, *17*, 8221 – 8233.

[58] Y. M. Choi-Sledeski, R. Kearney, G. Poli, H. Pauls, C. Gardner, Y. Gong, M. Becker, R. Davis, A. Spada, G. Liang, V. Chu, K. Brown, D. Collussi, R. Leadley, Jr., S. Rebello, P. Moxey, S. Morgan, R. Bentley, C. Kasiewski, S. Maignan, J.-P. Guilloteau, V. Mikol, *J. Med. Chem.* **2003**, *46*, 681 – 684.

[59] See Ref. [39].

[60] M. J. Kochanny, M. Adler, J. Ewing, B. D. Griedel, E. Ho, R. Karanjawala, W. Lee, D. Lentz, A. M. Liang, M. M. Morrissey, G. B. Phillips, J. Post, K. L. Sacchi, S. T. Sakata, B. Subramanyam, R. Vergona, J. Walters, K. A. White, M. Whitlow, B. Ye, Z. Zhao, K. J. Shaw, *Bioorg. Med. Chem.* **2007**, *15*, 2127 – 2146.

[61] B. Ye, D. O. Arnaiz, Y.-L. Chou, B. D. Griedel, R. Karanjawala, W. Lee, M. M. Morrissey, K. L. Sacchi, S. T. Sakata, K. J. Shaw, S. C. Wu, Z. Zhao, M. Adler, S. Cheeseman, W. P. Dole, J. Ewing, R. Fitch, D. Lentz, A. Liang, D. Light, J. Morser, J. Post, G. Rumennik, B. Subramanyam, M. E. Sullivan, R. Vergona, J. Walters, Y.-X. Wang, K. A. White, M. Whitlow, M. J. Kochanny, *J. Med. Chem.* **2007**, *50*, 2967 – 2980.

[62] P. Zhang, W. Huang, L. Wang, L. Bao, Z. J. Jia, S. M. Bauer, E. A. Goldman, G. D. Probst, Y. Song, T. Su, J. Fan, Y. Wu, W. Li, J. Woolfrey, U. Sinha, P. W. Wong, S. T. Edwards, A. E. Arfsten, L. A. Clizbe, J. Kanter, A. Pandey, G. Park, A. Hutchaleelaha, J. L. Lambing, S. J. Hollenbach, R. M. Scarborough, B.-Y. Zhu, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2179 – 2185.

[63] L. M. Salonen, C. Bucher, D. W. Banner, W. Haap, J.-L. Mary, J. Benz, O. Kuster, P. Seiler, W. B. Schweizer, F. Diederich, *Angew. Chem.* **2009**, *121*, 825 – 828; *Angew. Chem. Int. Ed.* **2009**, *48*, 811 – 814.
[64] M. L. Quan, Q. Han, J. M. Fevig, P. Y. S. Lam, S. Bai, R. M. Knabb, J. M. Luettgen, P. C. Wong, R. R. Wexler, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1795 – 1798.
[65] W. R. Gould, T. B. McClanahan, K. M. Welch, S. M. Baxi, K. Saiya-Cork, L. Chi, T. R. Johnson, R. J. Leadley, *J. Thromb. Haemostasis* **2006**, *4*, 834 – 841.
[66] E. C. Giardino, B. J. Haertlein, L. de Garavilla, M. J. Costanzo, B. P. Damiano, P. Andrade-Gordon, B. E. Maryanoff, *Blood Coagulation Fibrinolysis* **2010**, *21*, 128 – 134.
[67] A. Kranjc, D. Kikelj, *Curr. Med. Chem.* **2004**, *11*, 2535 – 2547.
[68] H. Nar, M. Bauer, A. Schmid, J.-M. Stassen, W. Wienen, H. M. W. Priepke, I. K. Kauffmann, U. J. Ries, N. H. Hauel, *Structure* **2001**, *9*, 29 – 37.
[69] a) E. U. Graefe-Mody, U. Schühly, K. Rathgen, H. Stähle, J. M. Leitner, B. Jilma, *J. Thromb. Haemostasis* **2006**, *4*, 1502 – 1509; b) U. J. Ries, *Abstr. Pap. 226th ACS National Meeting (New York, NY)*, **2003**, MEDI-033.
[70] J. M. Leitner, B. Jilma, F. B. Mayr, F. Cardona, A. O. Spiel, C. Firbas, K. Rathgen, H. Stähle, U. Schühly, E. U. Graefe-Mody, *Clin. Pharmacol. Ther.* **2007**, *81*, 858 – 866.
[71] a) J. Z. Deng, D. R. McMasters, P. M. A. Rabbat, P. D. Williams, C. A. Coburn, Y. Yan, L. C. Kuo, S. D. Lewis, B. J. Lucas, J. A. Krueger, B. Strulovici, J. P. Vacca, T. A. Lyle, C. S. Burgey, *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4411 – 4416; b) J. Z. Deng, C. S. Burgey, R. M. A. Rabbat, S. D. Lewis, B. J. Lucas, J. A. Krueger, R. B. White, B. Wong, E. A. Lyle, D. R. McMasters, *Abstr. Pap. 227th ACS National Meeting* (Anaheim, USA), **2004**, MEDI-095.
[72] R. J. Young, D. Brown, C. L. Burns-Kurtis, C. Chan, M. A. Convery, J. A. Hubbard, H. A. Kelly, A. J. Pateman, A. Patikis, S. Senger, G. P. Shah, J. R. Toomey, N. S. Watson, P. Zhou, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2927 – 2930.
[73] M. Follmann, V. Wehner, J.-M. Altenburger, G. Lassalle, J.-P. Herault (Sanofi-Aventis Fr.), WO 2009/103439, **2009**.
[74] M. Follmann, V. Wehner, J. Meneyrol, J.-M. Altenburger, F. Petit, G. Lassalle, J.-P. Herault (Sanofi-Aventis Fr.), WO 2009/103440, **2009**.

